BPB Reports

Regular Article

Tapinarof, a Novel Topical Therapeutic Aryl Hydrocarbon Receptor Agonist, Suppresses Atopic Dermatitis-like Skin Inflammation in Mice

Toshiki Urashima,[a,*] Yoshiaki Katsuda,[a] Hiromi Yoshiuchi,[a] Shin Ebihara,[a] Yuichi Shinozaki,[a] Tomoko Kato,[a] Taishi Shimazaki,[b] Yuzo Yasui,[b] Yasuko Mera,[a] and Noriko Konishi[a]

[a]*Biological/Pharmacological Research Laboratories, Takatsuki Research Center, Central Pharmaceutical Research Institute, Japan Tobacco Inc., 1-1, Murasaki-cho, Takatsuki, Osaka 569-1125, Japan;* [b]*Toxicology Research Laboratories, Yokohama Research Center, Central Pharmaceutical Research Institute, Japan Tobacco Inc., 1-13-2, Fukuura, Kanazawa-Ku, Yokohama, Kanagawa 236-0004, Japan*

Received March 22, 2024; Accepted July 19, 2024

Tapinarof is a non-steroidal, small molecule aryl hydrocarbon receptor (AhR) agonist that is being developed for the treatment of atopic dermatitis (AD). AD is a chronic inflammatory skin disease mediated by type 2 helper T (Th2) cells, characterized by impaired epidermal differentiation and skin barrier function. Tapinarof has been reported to regulate target gene expression through activation of AhR, improve skin barrier function, and exhibit an antioxidant effect. In this study, we investigated the pharmacological properties of tapinarof and its efficacy in mice with AD-like skin inflammation induced by 2, 4-dinitrofluorobenzene (DNFB). Tapinarof induced the mRNA expression of CYP1A1, an indicator of AhR activation, induced the mRNA expression of NAD(P)H: quinone oxidoreductase (NQO1), an antioxidant enzyme in human peripheral blood mononuclear cells (PBMCs) stimulated with T-cell activators. It also suppressed the production of interleukin (IL)-4, a Th2 cytokine. In mice with AD-like dermatitis, topical administration of tapinarof promoted the expression of *Cyp1a1* and *Nqo1* in the skin. It suppressed IL-4 production, the ear swelling, and histopathological changes. Tapinarof also suppressed an increase in transepidermal water loss (TEWL), an indicator of skin barrier function. These results indicate that tapinarof suppresses AD-like skin dermatitis and suggest that a variety of pharmacological actions, including an antioxidant effect, inhibition of Th2 cytokine, and improvement in barrier function, are involved.

Key words tapinarof, atopic dermatitis, cytokine signaling, IL-4, aryl hydrocarbon receptor

INTRODUCTION

Atopic dermatitis (AD) is clinically characterized by skin inflammation, skin barrier dysfunction, and chronic pruritus.[1,2] Persistent intense itching, leading to sleeplessness, and social stigma due to the visible symptoms of skin lesions, markedly impact the patients' quality of life.[3] Immunologic abnormalities and abnormal skin barrier function are known to be involved in the pathogenesis of AD.[1,2] Under physiological conditions, homeostasis of the skin barrier function is regulated by the coordinated expression of barrier-related proteins such as filaggrin (FLG), loricrin (LOR), and involucrin (IVL), intercellular lipids, and corneodesmosomes in the granular and cornified layers.[4] Loss-of-function mutations or reduced expression of FLG causes decreased barrier function of the stratum corneum, which has been shown to be involved in the pathogenesis of AD.[4,5,6] In terms of immunologic abnormalities, type 2 helper T (Th2) cytokines, interleukin (IL)-4 and IL-13, are suggested to play a pivotal role in the pathogenesis of AD. Dupilumab, a monoclonal antibody that blocks the shared receptor component for IL-4 and IL-13, is remarkably effective against rashes and pruritus in AD.[7,8] In addition, dupilumab corrects increased expression of various cytokines (IL-31, etc.) and chemokines (CCL17, CCL22, etc.) involved in skin inflammation and pruritus, as well as decreased expression of various molecules involved in skin barrier function.[9] Based on these facts, Th2 cytokines are considered central to the pathogenesis of AD.

Aryl hydrocarbon receptor (AhR) functions as a chemical sensor, transducing external and internal stimuli into biological responses. Once activated by various endogenous and exogenous ligands, AhR translocates to the nucleus and forms a heterodimer with the AhR nuclear transporter. This complex binds to the xenobiotic response element (XRE) and regulates downstream gene transcription.[10] The chemical metabolizing enzymes, CYP1A1, CYP1A2, and CYP1B1 are upregulated, leading to rapid degradation of these ligands and maintenance of homeostasis of keratinization.[11] NAD(P)H: quinone

*To whom correspondence should be addressed. e-mail: toshiki.urashima@jt.com

oxidoreductase (NQO1), an antioxidant molecule, also has an XRE motif in the promoter region, and its expression is enhanced in response to AhR activation.[12] This serves as a mechanism to neutralize reactive oxygen species-induced oxidative stress associated with ligand degradation by CYP1A1.

Tapinarof is a fully synthesized small molecule originally derived from a natural product of bacterial symbionts of entomopathogenic nematodes.[13-17] Recent studies have revealed that tapinarof activates the AhR, thereby improving skin barrier function by increasing the expression of barrier-related proteins, such as FLG and IVL.[10] In addition, tapinarof has an antioxidant effect through activation of nuclear factor erythroid 2-related factor 2 (NRF2) and directly increases the expression of antioxidant enzyme, NQO1.[10] Furthermore, tapinarof has been reported to suppress the production of the inflammatory cytokine, IL-17A, and reduce psoriasis (Ps)-like skin inflammation in mice induced by imiquimod through AhR agonism.[10] Tapinarof has already been approved in the US as a drug for the treatment of Ps in adults.[18] It is also under development as a drug for the treatment of AD, and favorable therapeutic effects have been reported in clinical studies.[19,20,21]

The therapeutic effects of tapinarof on Ps have primarily been attributed to its antioxidant effect, enhancement of skin barrier function, and suppression of Th17 cytokines through the agonistic action of AhR.[10] On the other hand, the mechanisms of action of tapinarof responsible for therapeutic effects in AD remain unclear. In this study, we investigated the pharmacological properties and efficacy of tapinarof in AD *in vitro* and *in vivo*. While Th2 cytokines are considered crucial for the pathogenesis of AD, the impact on type 2 cytokines of tapinarof has not been reported. Thus, the inhibitory effect of tapinarof on Th2 cytokine production was examined using human peripheral blood mononuclear cells (PBMCs). In addition, the pharmacological effects of tapinarof have not been investigated in detail in AD-like animal models *in vivo*. Therefore, we aimed to evaluate the potential effects of tapinarof on skin inflammation using mice with AD-like dermatitis, and investigate whether it might have anti-Th2 inflammatory, antioxidant, and skin barrier improving effects.

MATERIALS AND METHODS

Compounds Tapinarof was obtained from Dermavant Sciences GmbH (Basel, Switzerland). FK506 monohydrate and ethanol (EtOH) were purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). Dimethyl sulfoxide (DMSO) and 2, 4-dinitrofluorobenzene (DNFB) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). For the *in vitro* experiments, tapinarof was dissolved in DMSO and diluted with the culture medium used in each experiment. For the *in vivo* experiments, both tapinarof and FK506 were dissolved in ethanol for topical administration.

***In Vitro* Study in Human PBMCs** Cryopreserved PBMCs (STEMCELL Technologies Inc. Vancouver, BC, Canada) were thawed and suspended in RPMI-1640 medium (Thermo Fisher Scientific Inc. Waltham, MA, USA) containing 10% fetal bovine serum, 100 IU/mL penicillin G, and 100 µg/mL streptomycin. The cells were plated at 2×10^5 cells/well in a 96-well plate and stimulated with 2.0×10^4 beads/well Dynabeads Human T-Activator CD3/CD28 (VERITAS Co., Tokyo, Japan) for 2 days, followed by culture in the presence or absence of tapinarof for 1 day at 37°C in 5% CO_2. After washing the cultured cells with Dulbecco's Phosphate Buffered Saline, they were lysed and the total RNA was purified using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the kit's instructions. For cytokine measurement, PBMCs were seeded in a similar manner and stimulated with 0.5 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Co., St. Louis, MO, USA) and 1.0 µg/mL phytohemagglutinin-L (PHA-L) (Roche Diagnostics GmbH, Mannheim, Germany) for 2 days in the presence or absence of tapinarof at 37°C in 5% CO_2. The supernatants were collected, and IL-4 was measured with a Human IL-4 Quantikine HS ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA). Cell viability was evaluated by CellTiter-Glo 2.0 Assay (Promega Co., Madison, WI, USA).

Animals Six-week-old female BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and used for experiments after a week of acclimation. All mice were maintained under specific pathogen-free conditions at a room temperature of 23 ± 3°C and air humidity of $55\%\pm15\%$ on a 12-h/12-h light/dark cycle. All experiments were conducted in accordance with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and in compliance with the Guidelines for Animal Experimentation of the Central Pharmaceutical Research Institute of Japan Tobacco, Inc. (Protocol No. 02800, Date: Jan 18, 2022).

***In Vivo* Study in Mice with AD-like Dermatitis** Hapten-induced chronic dermatitis was induced in female BALB/c mice as described below. The ears of the mice were treated with 25 µL of 0.15% DNFB dissolved in acetone/olive oil (3:1) on both sides of the left ears five times at intervals of 3 or 4 days over a period of 15 days. Tapinarof and FK506 were administered topically on both sides of the left ears at a volume of 20 µL per mouse (10 µL to one side of the ear) once daily from the day of the first DNFB application, for 15 days from Days 1 to 15. Ear thickness as an index of ear swelling was measured with a digital thickness gauge (Digimatic Indicator; Mitutoyo Co., Kawasaki, Japan) on Days 1, 2, 8, 9, 15, and 16 and expressed as the increase in thickness from the baseline measurement. The measurement of the transepidermal water loss (TEWL) was performed after the measurement of the ear thickness on Day 16 with a Tewameter (VAPO SCAN AS-VT100RS, Asahi Techno Lab., Ltd., Yokohama, Japan). After the final measurement, mice were anesthetized and euthanized, and their left ears were excised and divided into three sections for analysis of gene expression, cytokine measurements, and histological evaluation. Ear sections were homogenized, and total RNA was prepared using the RNeasy Mini Kit according to the manufacturer's instructions. IL-4 levels were assessed with a Mouse IL-4 Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA). The remaining pieces of the ears were fixed in 10% neutral-buffered formalin (FUJIFILM Wako Pure Chemical, Osaka, Japan). The tissues were embedded in paraffin wax and stained with hematoxylin and eosin (HE). Tissue specimens were prepared for pathological evaluation by the Biopathology Institute (Kokuto, Japan).

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) RT-qPCR was performed using the TaqMan™ RNA-to-Ct™ 1-Step Kit (Thermo Fisher Scientific Inc.) and QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific). All Taqman probes (*CYP1A1*: Hs 01054794_m1, *NQO1*: Hs 00168547_m1, glyceraldehyde 3-aphosphate dehydrogenase (*GAPDH*): Hs 99999905_m1, *Cyp1a1*: Mm

00487218_m1, *Nqo1*: Mm 01253561_m1, and β-actin (*Actb*): Mm 02619580_m1) were purchased from Thermo Fisher Scientific. Comparative cycle time was used to normalize the transcript levels to those of *GAPDH in vitro* and to *Actb in vivo*, respectively.

Pathological Examinations The central region of the HE-stained ear sections, which corresponded approximately to the site where the ear thickness was measured, was examined under a light microscope. The microscopic findings were graded based on the following criteria: infiltration of inflammatory cells into dermis (–=negative: no lesion, ±=very slight: infiltration of small numbers of inflammatory cells partially or sparsely, +=slight: infiltration of small numbers of inflammatory cells diffusely, 2+=moderate: infiltration of moderate numbers of inflammatory cells with apparent thickening of the ear due to the increase in number of inflammatory cells, and 3+=severe: infiltration of large numbers of the inflammatory cells with marked thickening of the ear due to the increase in number of inflammatory cells), acanthosis (–=negative: no thickening, ±=very slight: very slight or focal thickening of epidermis: hypertrophy of the epidermal cells is absent, +=slight: slight thickening of the epidermis: hypertrophy of the epidermal cells is very slight or not seen, 2+=moderate: moderate thickening of the epidermis with slight hypertrophy of the epidermal cells, and 3+=severe: marked thickening of the epidermis with marked hypertrophy of epidermal cells), crust (–=negative: no lesion, ±=very slight: very slight or focal crust formation on the epidermis, +=slight: slight and multifocal crust formation on the epidermis with erosion, 2+=moderate: moderate and multifocal to diffuse crust formation on the epidermis with erosion and/or ulcers, and 3+=severe: marked and diffuse crust formation on the epidermis with erosion and/or ulcers).

Statistical Analyses Data are expressed as the mean and standard deviation of the indicated number of samples. SAS System Version 9.4 (SAS Institute Inc., Cary, NC, USA) and EXSUS Version 10.1 (EPS Co., Tokyo, Japan) were used for the statistical analyses. For the relative mRNA expression values or cytokine measurements *in vitro*, the significance of differences between the vehicle group and the tapinarof group was evaluated using the Shirley-Williams test. A one-tailed significance level of 2.5% was used. For values in the *in vivo* study, the significance of differences between two groups was assessed by Student *t*-tests (for homoscedastic data) or Aspin-Welch *t*-tests (for heteroscedastic data), after homoscedasticity analysis by F-tests. Differences among multiple groups were analyzed by Dunnett tests (for homoscedastic data) or Steel tests (for heteroscedastic data) after homoscedasticity analysis by the Bartlett test. A two-tailed significance level of 5% was used.

RESULTS

Tapinarof Activates AhR Pathway and Upregulates Antioxidant Gene Expression in Human PBMCs Tapinarof has been shown to directly bind to AhR, promote its

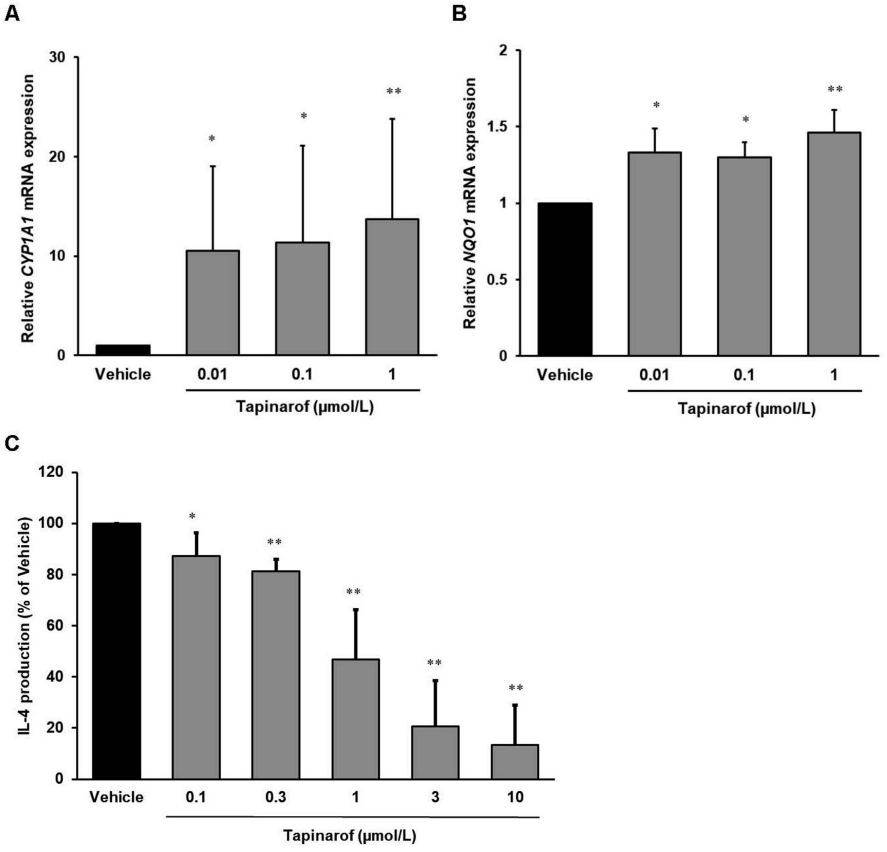

Fig. 1. Mechanism of Action and Effects of Tapinarof on Th2 Cytokine Levels in the Human PBMCs

Effects of tapinarof on the mRNA levels of *CYP1A1*(A) and *NQO1* (B) in human PBMCs, stimulated with anti-CD3/CD28 for T-cell activation. Data are presented as means + standard deviations (SD) (n = 4). A one-tailed significant difference versus vehicle group; * $p < 0.025$, ** $p < 0.005$ (Shirley-Williams test). ELISA kits were used to measure the IL-4 (C) level in the supernatant of human PBMCs stimulated with PHA-L/PMA, with or without tapinarof. Data are shown as mean + SD (n = 4). One-tailed significant difference versus vehicle group; *: $p < 0.025$, **: $p < 0.005$ (Shirley-Williams test).

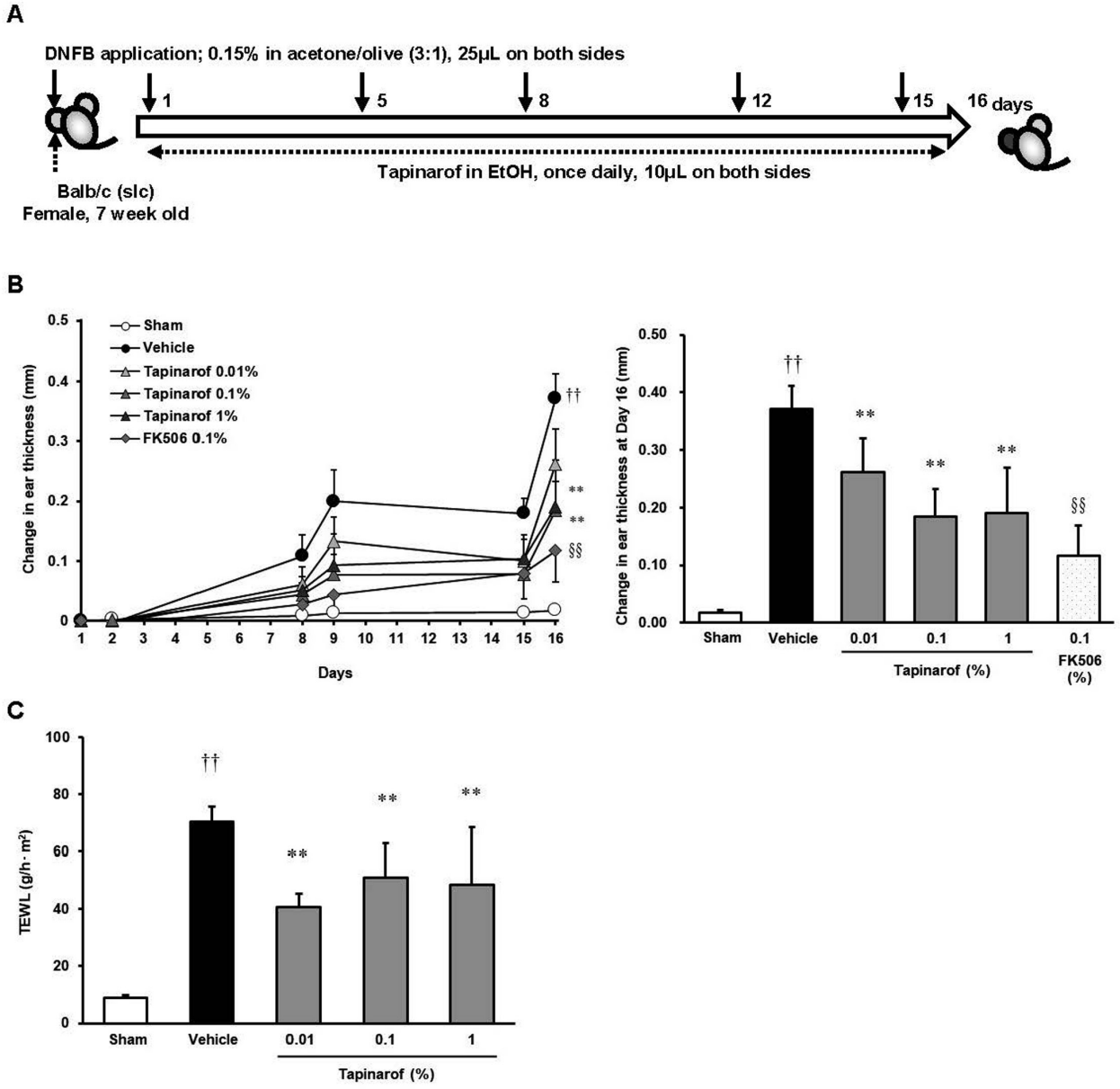

Fig. 2. Effects of Topical Administration of Tapinarof on Skin Inflammation in DNFB-Induced Dermatitis in Mice

(A) Mice received topical application of 0.15% DNFB in acetone/olive oil or vehicle (sham) on the ear at intervals of 3 or 4 days over 15 days. Vehicle (EtOH), tapinarof (0.01%, 0.1%, or 1%), or FK506 (0.1%) was administered topically once daily for 15 days. (B) Ear thickness was assessed on Days 1, 2, 8, 9, 15 and 16. Data are expressed as the increase in ear thickness from baseline. The results on day 16 are shown as the mean and SD (n = 10). Significant difference; ††, $p < 0.01$; sham vs. vehicle (Aspin-Welch *t*-test), §§, $p < 0.01$; vehicle vs. FK506 0.1% (Student *t*-test), **, $p < 0.01$; vehicle vs. tapinarof 0.01%, 0.1% and 1% (Dunnett test). (C) TEWL in the ear skin was measured on day 16 with a Tewameter. Data shown are mean + SD (n = 10). Significant difference; ††, $p < 0.01$; sham vs. vehicle (Aspin-Welch *t*-test), **, $p < 0.01$; vehicle vs. tapinarof 0.01%, 0.1%, and 1% (Steel test).

nuclear translocation, and to activate *CYP1A1* reporter activity.[10] It has also been shown to upregulate the gene expression of *CYP1A1* and exhibit antioxidant effects in human keratinocytes.[10] Given these results, we investigated the effect of tapinarof in human PBMCs on *CYP1A1* expression as a marker for AhR activation and on antioxidant molecule expression. Human PBMCs, stimulated with anti-CD3/CD28 antibody for 48 h, were treated with tapinarof at 0.01, 0.1, and 1 μmol/L. After 24 hours of treatment, the mRNA levels of *CYP1A1* and *NQO1* were significantly upregulated at concentrations of 0.01 μmol/L or higher compared to the vehicle group (Fig. 1A, B). These results suggest that tapinarof activates AhR pathway and antioxidant activity in human PBMCs.

Tapinarof Inhibits Th2 Cell Activation in Human PBMCs The pathogenesis of AD is primarily driven by Th2 cytokines, and inhibition of their production has shown therapeutic effects. Therefore, we investigated the effect of tapinarof on the production of Th2 cytokine *in vitro*. Upon stimulating human PBMCs with PHA-L and PMA, in the presence of tapinarof at concentrations of 0.1, 0.3, 1, 3, and 10 μmol/L for approximately 48 h, the concentrations of IL-4 decreased in a concentration-dependent manner (Fig. 1C). Reduction of the cell viability signal to less than 50% was observed at 10 μmol/L only (data not shown). These findings suggest that tapinarof inhibits Th2 cell activation in human PBMCs.

Tapinarof Inhibits AD-like Skin Inflammation in Mice Hapten-induced chronic dermatitis model is considered to involve Th2 cells and is a useful model of human AD.[22] To determine if tapinarof could reduce skin inflammation in dermatitis, we examined its effect on DNFB-induced dermatitis in mice (Fig. 2A). Repeated topical application of DNFB to mouse ears induced ear swelling at the second application and thereafter, and ear thickness increased in proportion to the number of exposures to DNFB (Fig. 2B). Topical administration of tapinarof at concentrations of 0.01%, 0.1%, and 1% was well tolerated (data not shown), and significantly inhibited the ear swelling at the concentrations of 0.01% and above (Fig. 2B). A 0.1% solution of FK506, used as reference, also inhibited the ear swelling. In addition, application of tapinarof resulted in a significant decrease in TEWL, an indicator of epidermal barrier function, compared to the vehicle group (Fig. 2C).

Tapinarof Attenuates Histopathological Changes in AD-like Skin Inflammation We further investigated the inhibitory effects of tapinarof on AD-like dermatitis in mice through histopathological examination of the ear skin. Severe inflammatory cell infiltration in the dermis, along with slight to moderate acanthosis and crust formation in the epidermis, were observed in vehicle group. In the FK506 group, the incidence or severity of the inflammatory cell infiltration, acanthosis, and crust formation was decreased as expected, whereas the sham group showed no findings. In the tapinarof group, the incidence or severity of the inflammatory cell infiltration, acanthosis, and crust formation was decreased dose-dependently from the lowest concentration (0.01%) (Table 1, Fig. 3, Figs. S1, S2). Therefore, based on these results, tapinarof was considered to attenuate AD-like dermatitis.

Tapinarof Activates AhR Pathway and Reduces IL-4 Level in Mice with AD-like Skin Dermatitis We investigated the pharmacological characteristics of tapinarof in this model. AhR agonism and subsequent antioxidant activation were assessed by measuring the mRNA expression of *Cyp1a1* and *Nqo1*, respectively. The results revealed a significant elevation from the lowest concentration of 0.01% in the tapinarof group compared to the vehicle group (Fig. 4A, B). The impact on inflammatory cell activation was evaluated by measuring IL-4 levels in ear skin. IL-4 levels were increased in the vehicle group after the final application of DNFB. Tapinarof significantly inhibited this cytokine elevation (Fig. 4C). Therefore, we suggest that inhibition of Th2 cytokine through activation of the AhR pathway is involved in the suppressive effect of tapinarof on AD-like dermatitis in mice.

DISCUSSION

Tapinarof is being developed for the treatment of AD and has attracted attention due to its effectiveness in treating AD in clinical trials.[19,20,21] Recent studies have reported that in normal human epidermal keratinocytes (NHEKs) and an immortalized human keratinocyte cell line, tapinarof regulates the expression of target genes involved in skin barrier function and antioxidant activity through AhR activation.[10, 23] Coal tar and glyteer, which have been used for the treatment of dermatitis for a long time, are known to activate the AhR and have skin barrier function-improving and antioxidant effects.[24,25] A photo-product of tryptophan and an endogenous AhR ligand, 6-formylindolo[3,2-b]carbazole, has also been shown to improve skin barrier dysfunction via AhR agonism in AD models.[6,26] From an immunological aspect, it has been reported that AhR activation by M50354 suppresses the differentiation of naïve Th cells into Th2 cells and decreases production of Th2 cytokines such as IL-4,[27] which is known to play an important role in the pathogenesis of AD. These previous findings suggested a hypothesis that tapinarof may ameliorate AD pathophysiology through AhR activation, in which anti-Th2 inflammatory effects, antioxidant effects, and improvements in skin barrier function are involved. In this study, we demonstrated that tapinarof (1) decreased IL-4 production in human PBMCs stimulated with T-cell activation inducers, (2) suppressed the AD-like skin inflammation in mice, and (3) activated AhR pathway, increased the expression of antioxidant molecule NQO1, and decreased IL-4 production in the ears of mice with AD-like skin inflammation, and (4) suppressed an increase in TEWL, an indicator of skin barrier function, in this model.

In the previous study, tapinarof was found to induce *CYP1A1* expression and inhibited cytokine (IL-17A) production in human T cells.[10] In this study, we first examined the *in vitro* pharmacological effects of tapinarof on the expression of AhR-related genes, *CYP1A1* and *NQO1*, as well as on the production of the Th2 cytokine, IL-4, in human PBMCs. To confirm the maximum effects of tapinarof, we used the concentrations of up to 1 μmol/L and 10 μmol/L for AhR-related gene expression and cytokine production, respectively, based on the results of the previous study.[10] As a result, tapinarof significantly induced *CYP1A1* and *NQO1* expression at 0.01 to 1 μmol/L, and the effects were almost at their maximum, which had no obvious dose-dependency, but showed dose-dependent reduction of IL-4 production at 0.1 to 10 μmol/L. It is known that the transcription of *CYP1A1* and *NQO1* is directly regulated by activated AhR.[10,12] On the other hand, suppression of IL-4 production has been reported to be mediated by an altered Th1/Th2 balance, and there was a shift reported in the effective concentration range of an AhR agonist between *Cyp1a1* promoter reporter activity and suppression of IL-4 production.[27] The observed discrepancy in the effective concentration range in this study may be due to differences in downstream regulation after AhR activation.

Next, we investigated the pharmacological effects of tapinarof on AD-like skin inflammation *in vivo* using mice with DNFB-induced chronic dermatitis. To our knowledge, there have been no previous reports examining the pharmacological properties of tapinarof in this model. Tapinarof significantly suppressed ear swelling at concentrations of 0.01% or higher and reduced histopathological inflammatory changes, including the incidence and severity of inflammatory cell infiltration in the dermis, acanthosis, and crust formation. These results demonstrated that tapinarof could inhibit AD-like skin inflammation in this model. Furthermore, the reduction in TEWL indicated that tapinarof improved skin barrier function, which aligns with previous findings that tapinarof increases the expression of barrier-related molecules. Additionally, tapinarof dose-dependently induced *Cyp1a1* and *Nqo1* mRNA expression in the ear, starting from 0.01%. Concurrently, tapinarof significantly decreased IL-4 levels in the ear tissue at concentrations of 0.01% or higher. This suggests that its Th2 cytokine suppressive effects, in addition to its barrier repair and antioxidant effects, may be involved in the suppression of AD pathogenesis by tapinarof.

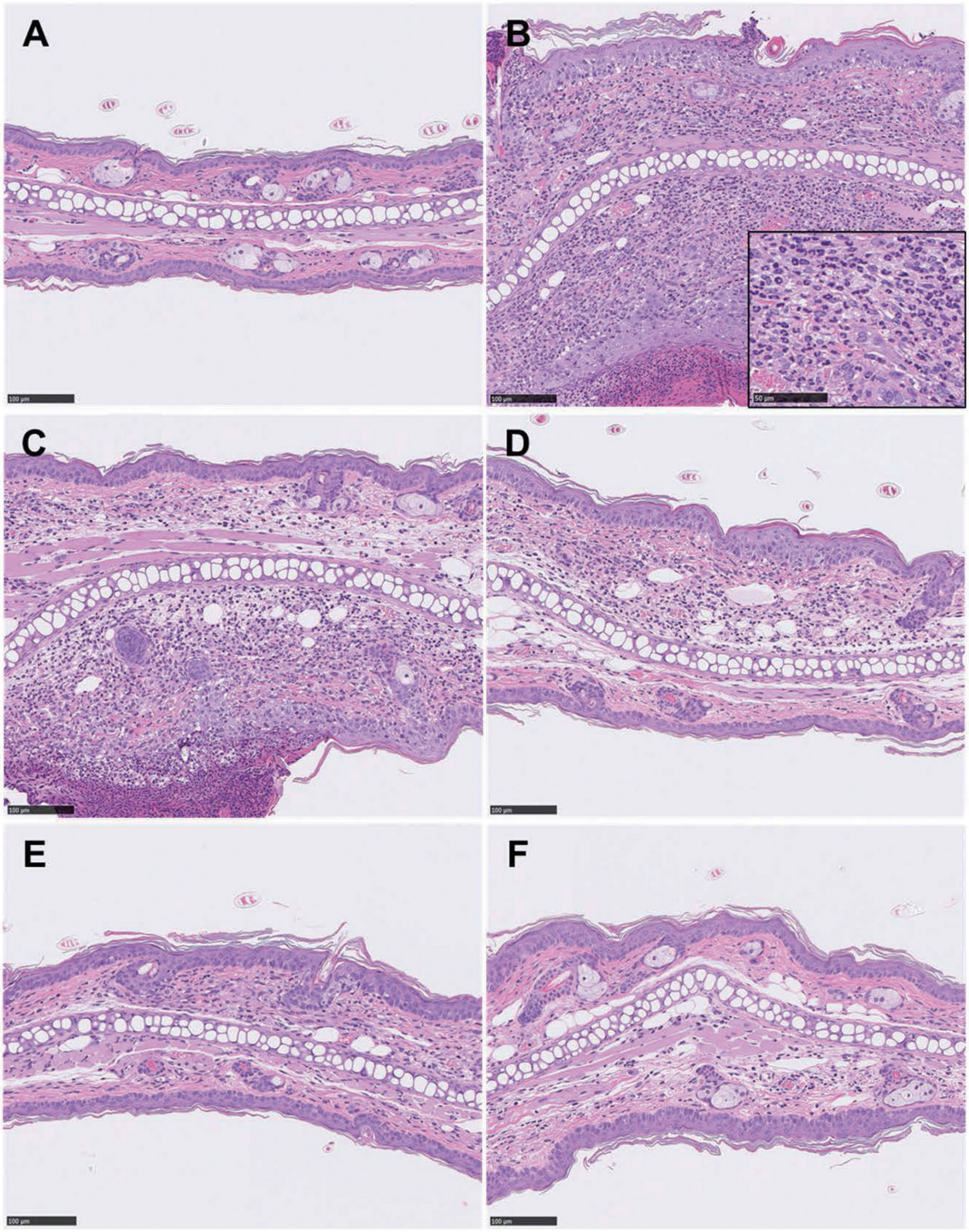

Fig. 3. Effect of Tapinarof on Inflammatory Cell Infiltration in the Dermis of the Ear Skin in DNFB-Induced Dermatitis in Mice

Histological analysis of the ear skin samples stained with HE was performed. Representative photomicrographs of sham, normal ear skin (A); vehicle, severe inflammatory cell infiltration in the dermis (B); 0.01% tapinarof, moderate (C); 0.1% tapinarof, slight (D); 1% tapinarof, very slight (E), and 1% FK506, very slight inflammatory cell infiltration in the dermis (F) are shown. Bar = 100 μm (inside B enlarged view: Bar = 50 μm).

The expression levels of *Cyp1a1* and *Nqo1* in the vehicle group were notably lower compared to the sham group, consistent with reports of reduced *CYP1A1* expression in keratinocytes of AD patients.[28] Moreover, increased AhR expression has been reported in the skin of AD patients.[29,30] These suggests that a deficiency in physiological ligands for AhR in the Th2 environment of AD and subsequent inactivation of the AhR pathway, possibly contributes to the exacerbation of AD pathology. Tapinarof restored *Cyp1a1* and *Nqo1* expression levels to sham levels at concentrations as low as 0.01%, indicating that even low-dose application of tapinarof could reach sufficient concentrations in the ear tissue to be effective in suppressing AD symptoms. This restoration at low concentration may explain why the inhibitory effects on IL-4 production, ear swelling, and TEWL reduction showed a saturation tendency at concentrations of 0.01% or higher, diverging from the dose-

Table 1. Effects of Tapinarof and FK506 on the Histopathological Changes in the Ear Skin (Microscopic Findings)

Organ	Findings	Group Concentration (%) Number of animals	Sham 10	Vehicle 10	Tapinarof 0.01% 10	Tapinarof 0.1% 10	Tapinarof 1% 10	FK506 0.1% 10
Skin (ear)			<10>	<10>	<10>	<10>	<10>	<10>
	Infiltration, inflammatory cells, dermis	–	10	0	0	0	1	2
		±	0	0	0	0	2	3
		+	0	0	3	5	2	4
		2+	0	0	6	5	5	1
		3+	0	10	1	0	0	0
	Acanthosis	–	10	10	0	0	2	3
		±	0	0	3	4	1	4
		+	0	7	6	6	7	3
		2+	0	3	1	0	0	0
		3+	0	0	0	0	0	0
	Crust	–	10	0	0	0	2	4
		±	0	0	4	2	2	4
		+	0	6	6	8	6	2
		2+	0	4	0	0	0	0
		3+	0	0	0	0	0	0

<>, Numbers of animals examined
-, No abnormal findings
±, Very slight; +, Slight; 2+, Moderate; 3+, Severe

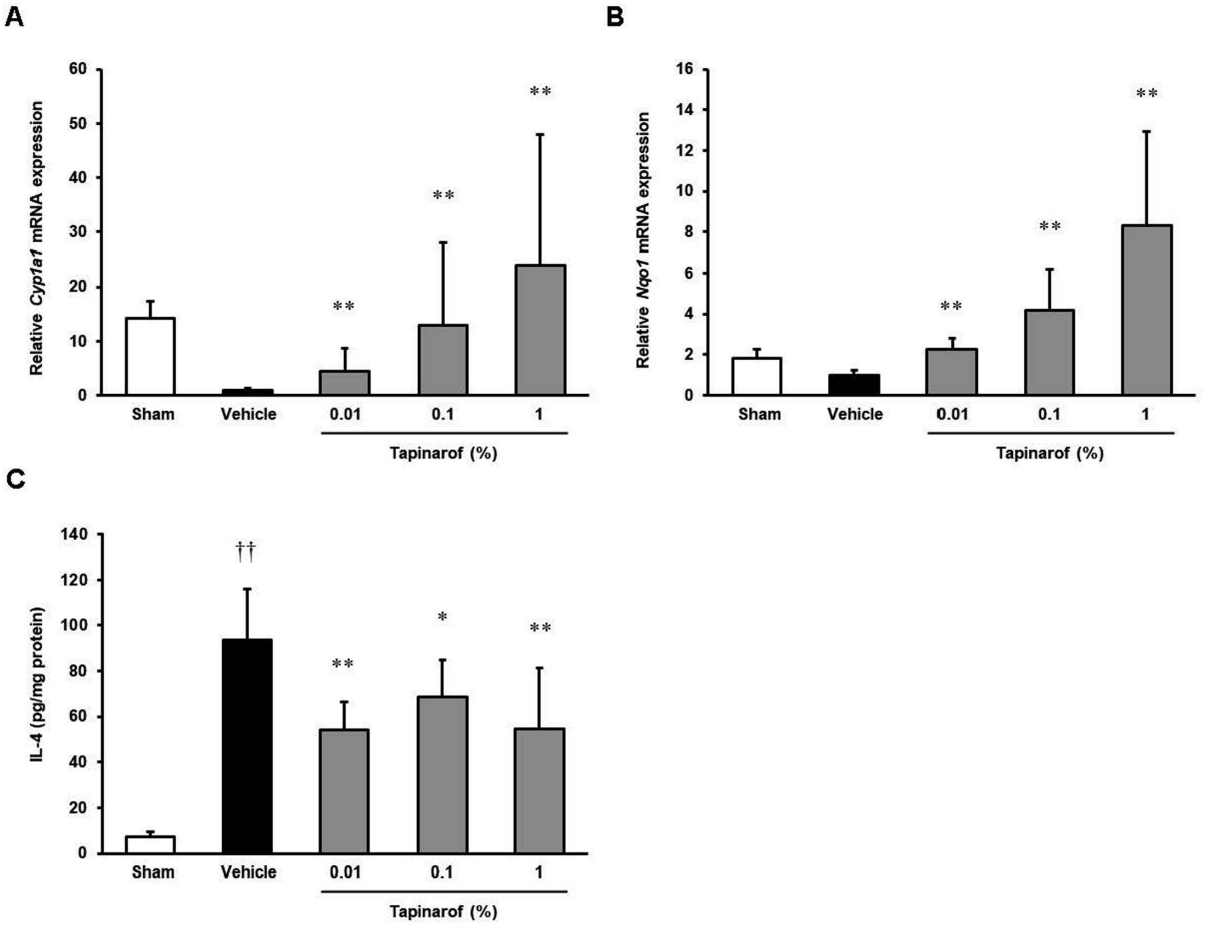

Fig. 4. Effects of Topical Administration of Tapinarof on AhR Activity and IL-4 Levels in DNFB-Induced Dermatitis in Mice

The relative mRNA expression of *Cyp1a1* (A) and *Nqo1* (B) in the ear tissue on day 16 is shown as fold change relative to vehicle control. Data are expressed as mean + SD (n = 10). Significant difference; **, $p < 0.01$ (Dunnett test). (C) ELISA kit was used to measure the IL-4 levels in the ear tissue on day 16. Data are shown as mean + SD (n = 10). Significant difference; ††, $p < 0.01$; sham vs. vehicle (Aspin-Welch *t*-test), *, $p < 0.05$; vehicle vs. tapinarof 0.1%, **, $p < 0.01$; vehicle vs. tapinarof 0.01% and 1% (Dunnett test).

dependent changes in *Cyp1a1* and *Nqo1* expression induction.

On the other hand, there were some limitations in the present study. In the evaluation of the DNFB-induced chronic dermatitis model this time, we focused on detecting the IL-4 levels in the ear tissue, and the effect of tapinarof on other Th2 cytokines such as IL-13 was not evaluated. In the previous report, IL-13 was measured 4 hours after the last DNFB application.[31] This condition was set based on the data that IL-13 levels in the ear peak 4 to 6 hours after the last application and decrease at 24 hours, whereas 24 hours was optimal for IL-4 measurement. IL-13 is known to be regulated by a negative feedback mechanism through its high-affinity binding to IL-13Rα2, a decoy receptor.[32,33] The difference in time course between IL-4 and IL-13 may reflect this negative feedback mechanism. Furthermore, in this study, we did not investigate the AhR dependency in the suppression of dermatitis, improvement of skin barrier function, antioxidant effects, or IL-4 suppression effects in the DNFB-induced chronic dermatitis model. Previous studies using AhR knockout mice have demonstrated that the suppression of imiquimod-induced dermatitis and other inflammatory cytokines is AhR-dependent. Therefore, further investigations are required to examine the AhR dependency using AhR KO mice or knockdown experiments to elucidate the precise mechanisms underlying tapinarof's effects.

AD is a chronic inflammatory skin disease clinically characterized by skin inflammation, skin barrier dysfunction, and chronic pruritus.[1,2] The current mainstay of treatment is the use of steroids and FK506, a calcineurin inhibitor, which exert their therapeutic effects mainly through their anti-inflammatory properties.[34] As described above, tapinarof has demonstrated improvements in skin barrier function and antioxidant effects. Due to its different mechanisms of action compared to approved drugs, tapinarof may be beneficial for non-responders or poor-responders to existing drugs. It has been shown that tapinarof enhanced Janus kinase (JAK) inhibitor-induced regulation of FLG and LOR expression in NHEKs.[23] Further study of the effects of combination treatment should be conducted. Additionally, recent clinical studies showed that tapinarof significantly improves pruritus.[21] It is known that lymphocytes enhance IgE production via IL-4Rα/γC receptor-JAK1/JAK3-STAT6 axis in response to IL-4.[11] It is also reported that activation of the AhR inhibits IL-4-induced IL-33 expression in keratinocytes, a cytokine associated with pruritus in AD.[35] Recently, tapinarof was reported to upregulate the expression of semaphorin-3A, a nerve-repellent factor, in human keratinocytes.[36] These results could potentially explain a portion of the mechanisms of anti-pruritic effects of tapinarof in the clinical trials. Further studies should be conducted in the future for a comprehensive understanding of the mechanisms of tapinarof on pruritus in AD.

In conclusion, this study indicates that tapinarof not only improves skin barrier function and has antioxidant effects, but also suppresses type 2 inflammation, which may contribute to the amelioration of AD symptoms. These findings provide a new perspective on the pharmacological actions of tapinarof and position it as a promising novel topical treatment candidate for AD with different pharmacological properties from existing topical agents.

Acknowledgments We thank Yoshiki Yamasaki for his valuable advice in regard to statistical analyses and Mutsuyoshi Matsushita and Jun Nishiu for their valuable suggestions.

Conflict of interest This work was financially supported by Japan Tobacco Inc. T.U., Y.K., H.Y., S.E., Y.S., T.K., T.S., Y.Y., Y.M., and N.K. are employees of Japan Tobacco Inc.

REFERENCES

1) Furue M, Chiba T, Tsuji G, Ulzii D, Kido-Nakahara M, Nakahara T, Kadono T. Atopic dermatitis: immune deviation, barrier dysfunction, IgE autoreactivity and new therapies. *Allergol. Int.*, **66**, 398–403 (2017).
2) Yang G, Seok JK, Kang HC, Cho YY, Lee HS, Lee JY. Skin barrier abnormalities and immune dysfunction in atopic dermatitis. *Int. J. Mol. Sci.*, **21**, 2867 (2020).
3) Lewis-Jones S. Quality of life and childhood atopic dermatitis: the misery of living with childhood eczema. *Int. J. Clin. Pract.*, **60**, 984–992 (2006).
4) Egawa G, Kabashima K. Barrier dysfunction in the skin allergy. *Allergol. Int.*, **67**, 3–11 (2018).
5) Paternoster L, Standl M, Waage J, Baurecht H, Hotze M, Strachan DP, Curtin JA, Bønnelykke K, Tian C, Takahashi A, Esparza-Gordillo J, Alves AC, Thyssen JP, den Dekker HT, Ferreira MA, Altmaier E, Sleiman PM, Xiao FL, Gonzalez JR, Marenholz I, Kalb B, Yanes MP, Xu CJ, Carstensen L, Groen-Blokhuis MM, Venturini C, Pennell CE, Barton SJ, Levin AM, Curjuric I, Bustamante M, Kreiner-Møller E, Lockett GA, Bacelis J, Bunyavanich S, Myers RA, Matanovic A, Kumar A, Tung JY, Hirota T, Kubo M, McArdle WL, Henderson AJ, Kemp JP, Zheng J, Smith GD, Rüschendorf F, Bauerfeind A, Lee-Kirsch MA, Arnold A, *et al*.; Australian Asthma Genetics Consortium (AAGC). Multi-ancestry genome-wide association study of 21,000 cases and 95,000 controls identifies new risk loci for atopic dermatitis. *Nat. Genet.*, **47**, 1449–1456 (2015).
6) Tsuji G, Hashimoto-Hachiya A, Kiyomatsu-Oda M, Takemura M, Ohno F, Ito T, Morino-Koga S, Mitoma C, Nakahara T, Uchi H, Furue M. Aryl hydrocarbon receptor activation restores filaggrin expression via OVOL1 in atopic dermatitis. *Cell Death Dis.*, **8**, e2931 (2017).
7) Paller AS, Silverberg JI, Cork MJ, Guttman-Yassky E, Lockshin B, Irvine AD, Kim MB, Kabashima K, Chen Z, Lu Y, Bansal A, Rossi AB, Shabbir A. Efficacy and safety of dupilumab in patients with erythrodermic atopic dermatitis. A Post Hoc Analysis of 6 Randomized Clinical Trials. *JAMA Dermatol.*, **159**, 255–266 (2023).
8) Worm M, Francuzik W, Kraft M, Alexiou A. Modern therapies in atopic dermatitis: biologics and small molecule drugs. *J. Dtsch. Dermatol. Ges.*, **18**, 1085–1092 (2020).
9) Guttman-Yassky E, Bissonnette R, Ungar B, Suárez-Fariñas M, Ardeleanu M, Esaki H, Suprun M, Estrada Y, Xu H, Peng X, Silverberg JI, Menter A, Krueger JG, Zhang R, Chaudhry U, Swanson B, Graham NMH, Pirozzi G, Yancopoulos GD, D Hamilton JD. Dupilumab progressively improves systemic and cutaneous abnormalities in patients with atopic dermatitis. *J. Allergy Clin. Immunol.*, **143**, 155–172 (2019).
10) Smith SH, Jayawickreme C, Rickard DJ, Nicodeme E, Bui T, Simmons C, Coquery CM, Neil J, Pryor WM, Mayhew D, Rajpal DK, Creech K, Furst S, Lee J, Wu D, Rastinejad F, Willson TM, Viviani F, Morris DC, Moore JT, Cote-Sierra J. Tapinarof is a natural AhR agonist that resolves skin inflammation in mice and humans. *J. Invest. Dermatol.*, **137**, 2110–2119 (2017).
11) Furue M. Regulation of filaggrin, loricrin, and involucrin by IL-4, IL-13, IL-17A, IL-22, AHR, and NRF2: pathogenic implications in atopic dermatitis. *Int. J. Mol. Sci.*, **21**, 5382 (2020).
12) Wang L, He X, Szklarz GD, Bi Y, Rojanasakul Y, Ma Q. The aryl hydrocarbon receptor interacts with nuclear factor erythroid 2-related factor 2 to mediate induction of NAD(P)H:quinoneoxidoreductase 1 by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Arch. Biochem. Biophys.*, **537**, 31–38 (2013).
13) Richardson WH, Schmidt TM, Nealson KH. Identification of an anthraquinone pigment and a hydroxystilbene antibiotic from Xenorhabdus

luminescens. *Appl. Environ. Microbiol.*, **54**, 1602–1605 (1988).
14) Li J, Chen G, Wu H, Webster JM. Identification of two pigments and a hydroxystilbene antibiotic from Photorhabdus luminescens. *Appl. Environ. Microbiol.*, **61**, 4329–4333 (1995).
15) Bissonnette R, Bolduc C, Maari C, Nigen S, Webster JM, Tang L, Lyle M. Efficacy and safety of topical WBI-1001 in patients with mild to moderate psoriasis: results from a randomized double-blind placebo-controlled, phase II trial. *J. Eur. Acad. Dermatol. Venereol.*, **26**, 1516–1521 (2012).
16) Bissonnette R, Chen G, Bolduc C, Maari C, Lyle M, Tang L, Webster J, Zhou Y. Efficacy and safety of topical WBI-1001 in the treatment of atopic dermatitis: results from a phase 2A, randomized, placebo-controlled clinical trial. *Arch. Dermatol.*, **146**, 446–449 (2010).
17) Bissonnette R, Poulin Y, Zhou Y, Tan J, Hong HC, Webster J, Ip W, Tang L, Lyle M. Efficacy and safety of topical WBI-1001 in patients with mild to severe atopic dermatitis: results from a 12-week, multicentre, randomized, placebo-controlled double-blind trial. *Br. J. Dermatol.*, **166**, 853–860 (2012).
18) Keam SJ. Tapinarof Cream 1%: first Approval. *Drugs*, **82**, 1221–1228 (2022).
19) Bissonnette R, Vasist LS, Bullman JN, Collingwood T, Chen G, Maeda-Chubachi T. Systemic pharmacokinetics, safety, and preliminary efficacy of topical AhR agonist tapinarof: results of a phase 1 study. *Clin. Pharmacol. Drug Dev.*, **7**, 524–531 (2018).
20) Paller AS, Stein Gold L, Soung J, Tallman AM, Rubenstein DS, Gooderham M. Efficacy and patient-reported outcomes from a phase 2b, randomized clinical trial of tapinarof cream for the treatment of adolescents and adults with atopic dermatitis. *J. Am. Acad. Dermatol.*, **84**, 632–638 (2021).
21) Silverberg JI, Boguniewicz M, Quintana FJ, Clark RA, Gross L, Hirano I, Tallman AM, Brown PM, Fredericks D, Rubenstein DS, McHale KA. Tapinarof validates the aryl hydrocarbon receptor as a therapeutic target: A clinical review. *J. Allergy Clin. Immunol.*, **154**, 1-10 (2024).
22) Inagaki N, Nagai H. Analysis of the mechanism for the development of allergic skin inflammation and the application for its treatment:mouse models for the development of remedies for human allergic dermatitis. *J. Pharmacol. Sci.*, **110**, 251–259 (2009).
23) Vu YH, Hashimoto-Hachiya A, Takemura M, Yumine A, Mitamura Y, Nakahara T, Furue M, Tsuji G. IL-24 negatively regulates keratinocyte differentiation induced by tapinarof, an aryl hydrocarbon receptor modulator: implication in the treatment of atopic dermatitis. *Int. J. Mol. Sci.*, **21**, 9412 (2020).
24) van den Bogaard EH, Bergboer JG, Vonk-Bergers M, van Vlijmen-Willems IM, Hato SV, van der Valk PG, Schröder JM, Joosten I, Zeeuwen PL, Schalkwijk J. Coal tar induces AHR-dependent skin barrier repair in atopic dermatitis. *J. Clin. Invest.*, **123**, 917–927 (2013).
25) Takei K, Mitoma C, Hashimoto-Hachiya A, Uchi H, Takahara M, Tsuji G, Kido-Nakahara M, Nakahara T, Furue M. Antioxidant soybean tar Glyteer rescues T-helper-mediated downregulation of filaggrin expression via aryl hydrocarbon receptor. *J. Dermatol.*, **42**, 171–180 (2015).
26) Kiyomatsu-Oda M, Uchi H, Morino-Koga S, Furue M. Protective role of 6-formylindolo[3,2-b]carbazole (FICZ), an endogenous ligand for arylhydrocarbon receptor, in chronic mite-induced dermatitis. *J. Dermatol. Sci.*, **90**, 284–294 (2018).
27) Negishi T, Kato Y, Ooneda O, Mimura J, Takada T, Mochizuki H, Yamamoto M, Fujii-Kuriyama Y, Furusako S. Effects of aryl hydrocarbon receptor signaling on the modulation of TH1/TH2 balance. *J. Immunol.*, **175**, 7348–7356 (2005).
28) Proper SP, Dwyer AT, Appiagyei A, Felton JM, Ben-Baruch Morgenstern N, Marlman JM, Kotliar M, Barski A, Troutman TD, Rothenberg ME, Mersha TB, Azouz NP. Aryl hydrocarbon receptor and IL-13 signaling crosstalk in human keratinocytes and atopic dermatitis. *Front. Allergy*, **5**, 1323405 (2024).
29) Hong CH, Lee CH, Yu HS, Huang SK. Benzopyrene, a major polyaromatic hydrocarbon in smoke fume, mobilizes Langerhans cells and polarizes Th2/17 responses in epicutaneous protein sensitization through the aryl hydrocarbon receptor. *Int. Immunopharmacol.*, **36**, 111–117 (2016).
30) Furue M, Hashimoto-Hachiya A, Tsuji G. Aryl Hydrocarbon Receptor in Atopic Dermatitis and Psoriasis. *Int. J. Mol. Sci.*, **20**, 5424 (2019).
31) Tanimoto A, Shinozaki Y, Yamamoto Y, Katsuda Y, Taniai-Riya E, Toyoda K, Kakimoto K, Kimoto Y, Amano W, Konishi N, Hayashi M. A novel JAK inhibitor JTE-052 reduces skin inflammation and ameliorates chronic dermatitis in rodent models: comparison with conventional therapeutic agents. *Exp. Dermatol.*, **27**, 22–29 (2018).
32) Ulzii D, Kido-Nakahara M, Nakahara T, Tsuji G, Furue K, Hashimoto-Hachiya A, Furue M. Scratching counteracts IL-13 signaling by upregulating the decoy receptor IL-13Rα2 in keratinocytes. *Int. J. Mol. Sci.*, **20**, 3324 (2019).
33) Furue M, Ulzii D, Nakahara T, Tsuji G, Furue K, Hashimoto-Hachiya A, Kido-Nakahara M. Implications of IL-13Rα2 in atopic skin inflammation. *Allergol. Int.*, **69**, 412–416 (2020).
34) Pariser D. Topical corticosteroids and topical calcineurin inhibitors in the treatment of atopic dermatitis: focus on percutaneous absorption. *Am. J. Ther.*, **16**, 264–273 (2009).
35) Tsuji G, Hashimoto-Hachiya A, Yen VH, Miake S, Takemura M, Mitamura Y, Ito T, Murata M, Furue M, Nakahara T. Aryl hydrocarbon receptor activation downregulates IL-33 expression in keratinocytes via ovo-like 1. *J. Clin. Med.*, **9**, 891 (2020).
36) Tsuji G, Yumine A, Yamamura K, Takemura M, Kido-Nakahara M, Ito T, Nakahara T. The Therapeutic Aryl Hydrocarbon Receptor-Modulating Agent Tapinarof Regulates SEMA3A Expression in Human Keratinocytes through NRF2. *J. Invest. Dermatol.*, **144**, 710–713.e8 (2024).