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ORIGINAL ARTICLE

Antimycotics suppress the *Malassezia* extract-induced production of CXC chemokine ligand 10 in human keratinocytes

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ABSTRACT

***Malassezia*, a lipophilic yeast, exacerbates atopic dermatitis. *Malassezia* products can penetrate the disintegrated stratum corneum and encounter subcorneal keratinocytes in the skin of atopic dermatitis patients. Type 1 helper T (Th1) cells infiltrate chronic lesions with atopic dermatitis, and antimycotic agents improve its symptoms. We aimed to identify *Malassezia*-induced chemokines in keratinocytes and examine whether antimycotics suppressed this induction. Normal human keratinocytes were incubated with a *Malassezia restricta* extract and antimycotics. Chemokine expression was analyzed by enzyme-linked immunosorbent assays and real-time polymerase chain reaction. Signal transducer and activator of transcription (STAT)1 activity was examined by luciferase assays. The tyrosine-phosphorylation of STAT1 was analyzed by western blotting. The *M. restricta* extract increased the mRNA and protein expression of Th1-attracting CXC chemokine ligand (CXCL)10 and STAT1 activity and phosphorylation in keratinocytes, which was suppressed by a Janus kinase inhibitor. The antimycotics itraconazole, ketoconazole, luliconazole, terbinafine, butenafine and amorolfine suppressed *M. restricta* extract-induced CXCL10 mRNA and protein expression and STAT1 activity and phosphorylation. These effects were similarly induced by 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), a prostaglandin D_2 metabolite. Antimycotics increased the release of 15d-PGJ_2 from keratinocytes. The antimycotic-induced suppression of CXCL10 production and STAT1 activity was counteracted by a lipocalin-type prostaglandin D synthase inhibitor. The antimycotics itraconazole, ketoconazole, luliconazole, terbinafine, butenafine and amorolfine may suppress the *M. restricta*-induced production of CXCL10 by inhibiting STAT1 through an increase in 15d-PGJ_2 production in keratinocytes. These antimycotics may block the Th1-mediated inflammation triggered by *Malassezia* in the chronic phase of atopic dermatitis.**

Key words: **antimycotic, atopic dermatitis, CXC chemokine ligand 10, keratinocyte, *Malassezia*.**

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory dermatosis characterized by xerosis, eczema and pruritus.[1] Skin barrier dysfunction, environmental agents and immune system abnormalities are implicated in the pathogenesis of AD.[1] Patients suffering from AD often carry mutations in the gene encoding filaggrin (*FLG*), which is important for the formation of the cornified envelope.[1] The *FLG* gene mutation results in reduced levels of filaggrin breakdown products that maintain hydration and acidification of the stratum corneum (SC), leading to a pH increase.[1] The increased pH enhances the activity of proteases that cleave corneodesmosomes, which lock corneocytes together in the SC, and decreases the activity of lipid biosynthetic enzymes, leading to a reduction of ceramide in the SC intercellular space.[1] Thus, in the skin of AD patients, the integrity of the SC is disrupted, allowing for irritant, allergen or microorganism permeation.[1] Among these microorganisms, commensal lipophilic yeasts of the *Malassezia* spp. colonize the seborrheic regions of human skin, such as the head, neck and upper trunk, and they may be involved in the development of AD.[2] Several *Malassezia*-derived allergens that elicit specific immunoglobulin (Ig)E responses and T-cell reactivity in AD patients have been cloned.[2] The addition of *Malassezia* extracts or infection with *Malassezia* in keratinocytes *in vitro* induces the production of cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, thymic stromal lymphopoietin (TSLP), IL-8, IL-10, IL-4, IL-5, IL-13 and antimicrobial peptide human β-defensin-2.[3–5] Toll-like receptor (TLR)2 may be involved in the *Malassezia*-induced production of IL-8 or

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human β-defensin-2,[3] and lysophosphatidic acid receptor (LPA)1 or LPA3 may be involved in *Malassezia*-induced TSLP production.[5]

Antimycotic agents are reportedly therapeutically effective in AD patients.[6,7] One possible mechanism is that antimycotics suppress *Malassezia* colonization through fungicidal effects.[7] Another possible mechanism involves antimycotic anti-inflammatory effects on keratinocytes or lymphocytes.[8,9] We recently found that the antimycotics itraconazole, ketoconazole, luliconazole, terbinafine, butenafine and amorolfine suppress TSLP production in polyinosinic–polycytidylic acid plus IL-4-stimulated human keratinocytes through upregulation of the cyclopentenone prostanoid 15-deoxy-Δ-12,14-prostaglandin J_2 (15d-PGJ_2).[10] 15d-PGJ_2 suppresses nuclear factor-κB (NF-κB) activation and NF-κB-dependent TSLP expression.[10]

Type 2 helper T (Th2) cells predominantly infiltrate the dermis of acute AD lesions, while Th1 and Th2 cells infiltrate chronic lesions.[11] Th22 or type 22 cytotoxic T cells also act as effector T cells in chronic AD lesions, while Th17 cells may transiently infiltrate acute lesions.[12] CC chemokine ligand (CCL)17/20, CXC chemokine ligand (CXCL)10, CCL20 and CCL27 are ligands for CC chemokine receptor (CCR)4, CXC chemokine receptor (CXCR) 3, CCR6 and CCR10, respectively, and chemoattract Th2, Th1, Th17 and Th22 cells, respectively.[11,12] *Malassezia*-derived products may penetrate the disintegrated SC of AD patients, contact the subcorneal keratinocytes, and trigger the production of certain chemokines that attract effector T cells. In this study, we first identified the types of T-cell-attracting chemokines produced by *Malassezia* extract-treated human keratinocytes *in vitro*. We found that the *Malassezia restricta* extract induced the production of CXCL10, a Th1-attracting chemokine. We next examined if antimycotics suppressed *M. restricta*-induced CXCL10 production in a 15d-PGJ_2-related manner.

METHODS

Reagents

Helenalin and Janus kinase (JAK) inhibitor I were purchased from Calbiochem (La Jolla, CA, USA). Recombinant human TNF-α, IL-17, interferon (IFN)-γ and monoclonal mouse antihuman TLR2 antibody were purchased from R&D Systems (Minneapolis, MN, USA). 15d-PGJ_2, PGE_2, $PGF_{2\alpha}$, rosiglitazone, BW245C, 13,14-dihydro-15-keto-PGD_2, 9,10-dihydro-15d-PGJ_2 (CAY10410), AT-56 and HQL-79 were from Cayman Chemical (Ann Arbor, MI, USA). VPC32183 was from Avanti Polar Lipids (Alabaster, AL, USA). The *M. restricta* extract was prepared from strain NBRC103918 as previously described,[13] dissolved in phosphate-buffered saline and stored at −20°C until use. Itraconazole, ketoconazole, fluconazole, voriconazole and terbinafine hydrochloride were from Wako Pure Chemical (Osaka, Japan). Luliconazole, butenafine hydrochloride and amorolfine hydrochloride were donated by POLA PHARMA (Tokyo, Japan), Kaken Pharmaceutical (Tokyo, Japan) and Kyorin Pharmaceutical (Tokyo, Japan), respectively. The antimycotics were dissolved in 100 mmol/L dimethylsulfoxide (DMSO) and subsequently diluted in the experimental media to yield the final concentrations. The control vehicle (DMSO) concentration was 0.1% (v/v).

Keratinocyte culture

Human neonatal foreskin keratinocytes were purchased from Clonetics (Walkersville, MD, USA). The keratinocytes were cultured in a keratinocyte growth medium (Clonetics) containing keratinocyte basal medium (KBM) supplemented with 0.5 μg/mL hydrocortisone, 5 ng/mL epidermal growth factor, 5 μg/mL insulin and 0.5% bovine pituitary extract. Cells in their third passage were used. Each experiment was performed four times using the same lot of keratinocytes from a single donor.

Enzyme-linked immunosorbent assay

Keratinocytes (5×10^4 cells/well) were seeded in triplicate in 24-well plates in 0.4 mL keratinocyte growth medium and allowed to adhere overnight. The cells were washed and incubated with supplement-free KBM for 24 h. The cells were washed and pre-incubated with DMSO or the indicated concentrations of antimycotics, prostanoids, helenalin, JAK inhibitor I, VPC32183 or anti-TLR2 antibody for 30 min and then incubated with the indicated concentrations of the *M. restricta* extract, TNF-α, IL-17 or IFN-γ in KBM for 48 h. The culture supernatants were analyzed by enzyme-linked immunoassay for CXCL9, CXCL10, CXCL11, CCL17, CCL20, CCL22, CCL27 (R&D Systems), 15d-PGJ_2 (Abnova, Taipei, Taiwan), PGD_2, TXB_2, PGE_2 or $PGF_{2\alpha}$ (Cayman Chemical) according to the manufacturers' instructions. In some experiments, the cells were pretreated with HQL-79 or AT-56 (each 100 μmol/L) for 30 min before the addition of antimycotics.

Real-time polymerase chain reaction

Keratinocytes were pre-incubated and then incubated for 24 h as described above. The total cellular RNA was extracted using RNeasy Mini kits (SABiosciences, Frederick, MD, USA). cDNA was synthesized using Superscript III First Strand synthesis kits (Invitrogen, Carlsbad, CA, USA). CXCL10 mRNA levels were quantified by a SYBR Green I gene expression assay (TaKaRa Bio, Tokyo, Japan). The mRNA levels of CXCL10, normalized to glyceraldehyde-3-phosphate dehydrogenase expression, are represented as the fold-change relative to the CXCL10 mRNA level in control keratinocytes treated with KBM alone.

Plasmids and transfection

A signal transducer and activator of transcription (STAT)1-responsive firefly luciferase vector containing tandem repeats of the IFN-γ activation sequence and a constitutive *Renilla* luciferase vector (SABiosciences) were mixed with FuGENE HD (Roche, Indianapolis, IN, USA) and added to the keratinocytes (3.0×10^4 cells/well) in 24-well plates. After 24 h, the cells were washed and incubated with KBM for another 24 h. Cells were pre-incubated with DMSO or the indicated concentrations of antimycotics, prostanoids or signal inhibitors for 30 min, followed by incubation with the indicated concentrations of the *M. restricta* extract or IFN-γ. After 18 h, the firefly and *Renilla* luciferase activities in the cell extracts were quantified using

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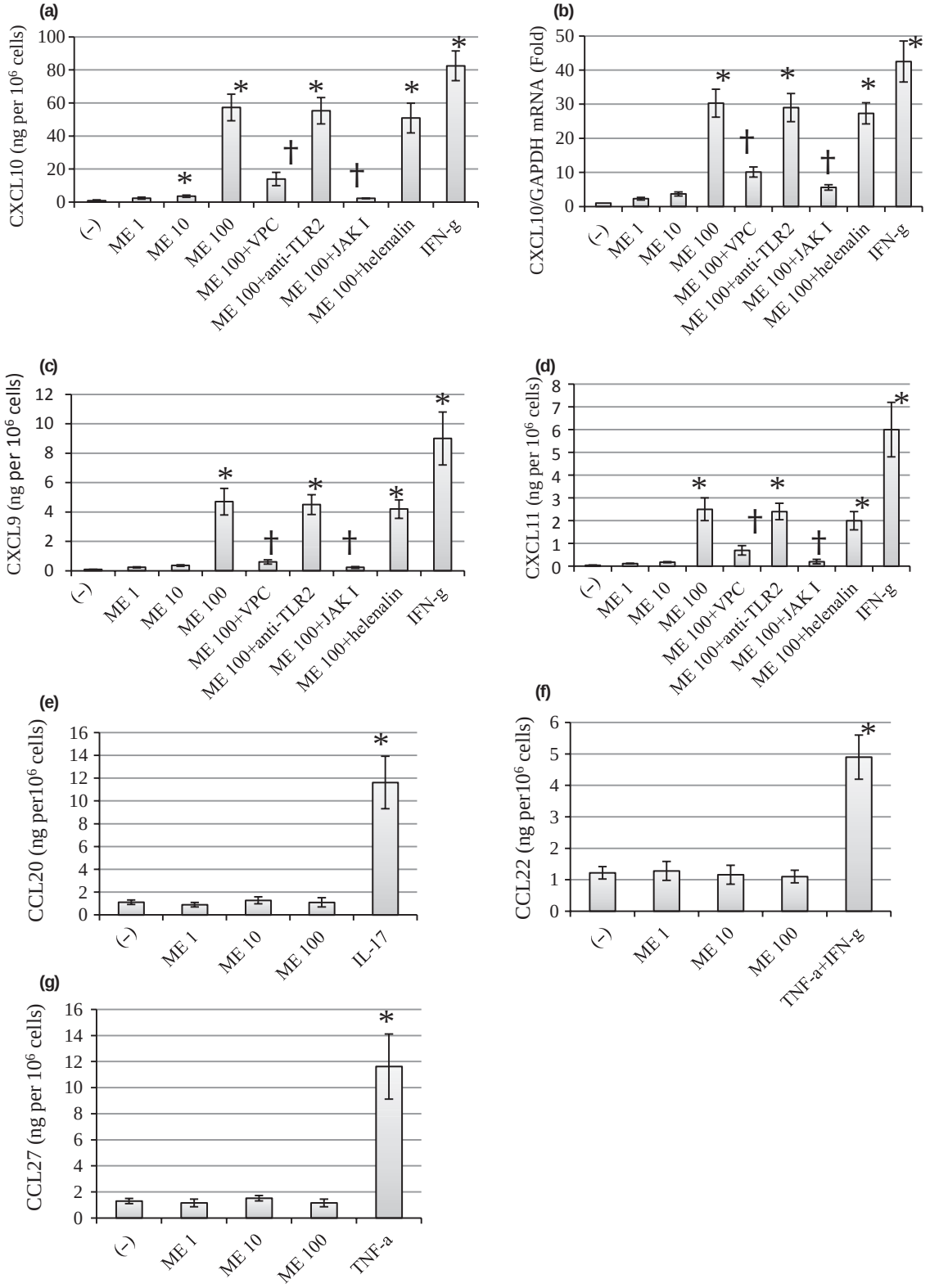

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Figure 1. Effects of the *Malassezia restricta* extract on the production of T-cell-attracting chemokines in keratinocytes. (a–d) Keratinocytes were pre-incubated with the vehicle (dimethylsulfoxide) or 20 μmol/L VPC32183 (VPC), 20 μg/mL anti-Toll-like receptor (TLR)2 antibody, 10 μmol/L Janus kinase (JAK) inhibitor I (JAK I) or 10 μmol/L helenalin for 30 min and then incubated with 1, 10 or 100 μg/mL *M. restricta* extract (ME) or 0.1 ng/mL interferon (IFN)-γ. CXC chemokine ligand (CXCL)10 (a), CXCL9 (c) and CXCL11 (d) secretion or CXCL10 mRNA levels (b) were analyzed at 48 or 24 h, respectively. (b) The CXCL10 mRNA levels normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are represented as the fold-change relative to the levels in control keratinocytes treated with keratinocyte basal medium (KBM) alone. (e,g) Keratinocytes were incubated with 1, 10 or 100 μg/mL ME or 10 ng/mL interleukin (IL)-17 (e), 10 ng/mL tumor necrosis factor (TNF)-α plus 10 ng/mL IFN-γ (f), or 10 ng/mL TNF-α (g). At 48 h, CCL20 (e), CCL22 (f) and CCL27 secretion (g) were analyzed. *$P < 0.05$ versus controls and †$P < 0.05$ versus 100 μg/mL ME by one-way ANOVA with Scheffé's test. Mean ± standard deviation ($n = 4$) is shown.

the Dual-Luciferase Assay System (Promega, Madison, WI, USA). The transcriptional activities of STAT1 are expressed as the ratio of firefly to *Renilla* luciferase activity.

Western blotting

Western blotting was performed as previously described.[14] In brief, keratinocytes were pre-incubated with DMSO or antimycotics or prostanoids for 30 min and then incubated with *M. restricta* extract for 15 min. Cells were lysed with lysis buffer; the resultant lysates were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked and probed using the primary antibodies anti-phospho STAT1 (Tyr701) or anti-pan STAT1 (Cell Signaling Technology, Boston, MA, USA). Appropriate secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added, and the immunoreactivity was visualized using LumiGLO (Cell Signaling Technology). Protein bands were analyzed by densitometry. The levels of phospho-STAT1 were normalized against pan-STAT1 and represented as the fold-change relative to the level in control keratinocytes treated with KBM alone.

Statistical analyses

All statistical analyses were performed using Ekuseru-Toukei 2012 (Social Survey Research Information, Tokyo, Japan). One-way ANOVA with Scheffé's test was used in Figures 1, 2(a), 7 and 8, and one-way ANOVA with Dunnett's test was used for the data in Figures 3, 4 and 6(a–c). $P < 0.05$ was considered significant.

RESULTS

M. restricta extract induces CXCL10 production by activating STAT1 in keratinocytes

Malassezia restricta extract increased CXCL10 secretion (Fig. 1a) and mRNA levels (Fig. 1b) in keratinocytes, with the maximum increase elicited at 100 μg/mL. *M. restricta* extract-induced CXCL10 secretion (Fig. 1a) and mRNA expression (Fig. 1b) were suppressed by the LPA1/LPA3 antagonist VPC32183 and JAK inhibitor I, but not by the anti-TLR2 antibody or NF-κB inhibitor helenalin, indicating the involvement of LPA1 or LPA3 and the JAK/STAT pathway in CXCL10 induction. *M. restricta* extract also increased the secretion of CXCL9, CXCL11, the other Th1-attracting chemokines; the secretion was suppressed by VPC32183 and JAK inhibitor I, but not by the anti-TLR2 antibody or helenalin (Fig. 1c,d). However, the *M. restricta* extract did not induce CCL20 (Fig. 1e), CCL22 (Fig. 1f), or CCL27 (Fig. 1g) secretion. CCL17 levels were undetectable in the presence or absence of *M. restricta* extract.

The *CXCL10* gene contains STAT1 response elements that bind activated STAT1 to induce gene expression.[15] The tyrosine phosphorylation of STAT1 is required for its dimerization,

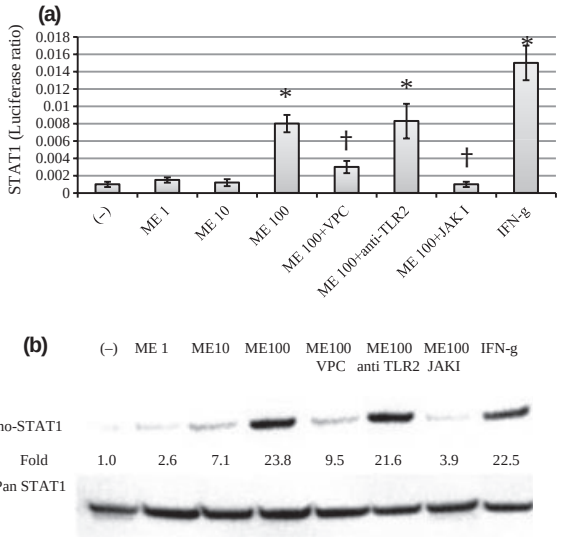

Figure 2. Effects of the *Malassezia restricta* extract on signal transducer and activator of transcription (STAT)1 activity (a) and phosphorylation (b) in keratinocytes. Keratinocytes, transfected with a STAT1-responsive firefly luciferase vector and *Renilla* luciferase vector (a) or untransfected (b), were pre-incubated with the vehicle (dimethylsulfoxide) or 20 μmol/L VPC32183 (VPC), 20 μg/mL anti-Toll-like receptor (TLR)2 antibody, or 10 μmol/L Janus kinase (JAK) inhibitor I (JAK I) for 30 min. Next, keratinocytes were incubated with 1, 10 or 100 μg/mL *M. restricta* extract (ME) or 0.1 ng/mL interferon (IFN)-γ. STAT1 transcriptional activity (a) or phospho-STAT1 and pan STAT1 levels (b) were analyzed at 18 h or 15 min, respectively. (a) The transcriptional activity of STAT1 is expressed as the ratio of the firefly to the *Renilla* luciferase activity. *$P < 0.05$ versus controls and †$P < 0.05$ versus 100 μg/mL ME by one-way ANOVA with Scheffé's test. Mean ± standard deviation ($n = 4$) is shown. (b) The phospho-STAT1 levels normalized to the levels of pan STAT1 are represented as the fold-change. The results represent four separate experiments.

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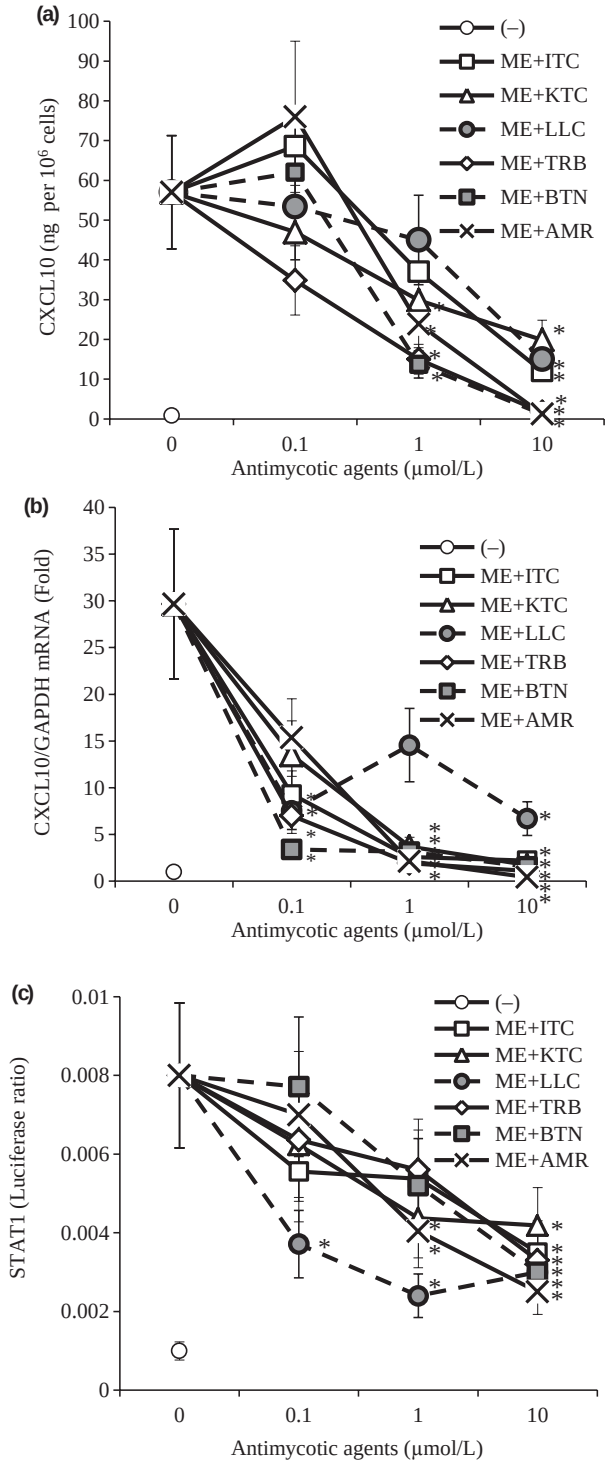

Figure 3. Effects of antimycotics on *Malassezia restricta* extract-induced CXC chemokine ligand (CXCL)10 secretion (a), CXCL10 mRNA expression (b) and signal transducer and activator of transcription (STAT)1 activity (c). Keratinocytes, untransfected (a,b) or transfected with a STAT1-responsive firefly luciferase vector and *Renilla* luciferase vector (c), were preincubated with the vehicle (dimethylsulfoxide) or the indicated concentrations of antimycotics for 30 min and then incubated with 100 μg/mL *M. restricta* extract. CXCL10 secretion (a), CXCL10 mRNA levels (b), or STAT1 activity (c) were analyzed at 48, 24 or 18 h, respectively. *$P < 0.05$ versus *M. restricta* extract alone by one-way ANOVA with Dunnett's test. Mean ± standard deviation ($n = 4$) is shown. ME, *M. restricta* extract; ITC, itraconazole; KTC, ketoconazole; LLC, luliconazole; TRB, terbinafine; BTN, butenafine; AMR, amorolfine.

nuclear translocation and DNA binding. We analyzed the effects of the *M. restricta* extract on STAT1 activities. The *M. restricta* extract enhanced STAT1-mediated transcription in keratinocytes. This response was suppressed by VPC32183 and JAK inhibitor I, but not by the anti-TLR2 antibody (Fig. 2a). The extract induced the tyrosine phosphorylation of STAT1. This response was suppressed by VPC32183 and JAK inhibitor I, but not by the anti-TLR2 antibody (Fig. 2b). Thus, *M. restricta* extract-induced phosphorylation and activation of STAT1 may involve LPA1/3 and JAK. These results suggest that the *M. restricta* extract induces CXCL10 production by activating the JAK/STAT1 pathway via LPA1/3 receptor engagement in keratinocytes.

Certain antimycotics suppress *M. restricta* extract-induced CXCL10 production by inhibiting STAT1 activity in keratinocytes

We next analyzed if antimycotics alter *M. restricta* extract-induced CXCL10 production in keratinocytes. When used at concentrations of less than 100 μmol/L, the antimycotics tested did not reduce the cell viability (>95% of controls), as examined by a Trypan blue dye exclusion test. *M. restricta* extract-induced CXCL10 secretion was suppressed by the antimycotics itraconazole, ketoconazole, luliconazole, terbinafine, butenafine and amorolfine in a dose-dependent manner (Fig. 3a). The *M. restricta* extract-induced increase in CXCL10 mRNA was also suppressed by these antimycotics (Fig. 3b), indicating transcriptional repression. We then examined if these antimycotics suppressed the STAT1 activities required for CXCL10 production. *M. restricta* extract-induced STAT1 activity was reduced in a dose-dependent manner by itraconazole, ketoconazole, luliconazole, terbinafine, butenafine and amorolfine (Fig. 3c). In contrast, the other antimycotics, fluconazole and voriconazole, did not suppress *M. restricta* extract-induced CXCL10 secretion, CXCL10 mRNA expression or STAT1 activity (Fig. 4).

Likewise, *M. restricta* extract-induced phosphorylation of STAT1 was suppressed by itraconazole, ketoconazole, luliconazole, terbinafine, butenafine and amorolfine, but not by fluconazole or voriconazole (Fig. 5). When keratinocytes pretreated with antimycotics were washed before the addition of *M. restricta* extract, STAT1 phosphorylation was still suppressed by the antimycotics (data not shown), indicating that

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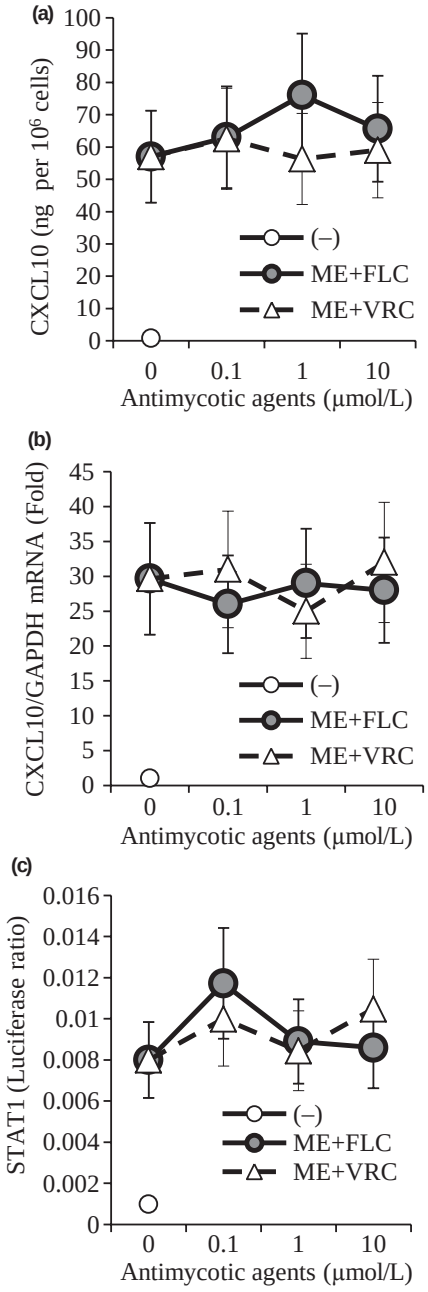

Figure 4. Effects of fluconazole or voriconazole on *Malassezia restricta* extract-induced CXC chemokine ligand (CXCL)10 secretion (a), CXCL10 mRNA expression (b) and signal transducer and activator of transcription (STAT)1 activity (c). Keratinocytes, untransfected (a,b) or transfected with STAT1-responsive firefly luciferase vector and *Renilla* luciferase vector (c), were pre-incubated with the vehicle (dimethylsulfoxide) or the indicated concentrations of fluconazole (FLC) or voriconazole (VRC) for 30 min and incubated with 100 μg/mL *M. restricta* extract (ME). CXCL10 secretion (a), CXCL10 mRNA levels (b), and STAT1 activities (c) were analyzed at 48, 24 and 18 h, respectively. Mean ± standard deviation (n = 4) is shown.

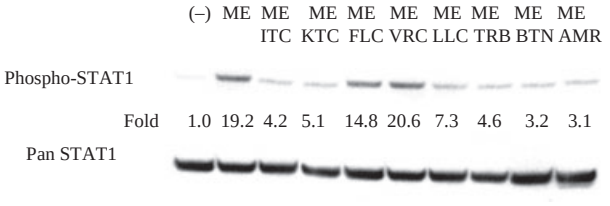

Figure 5. Effects of antimycotics on the *Malassezia restricta* extract-induced phosphorylation of signal transducer and activator of transcription (STAT)1. Keratinocytes were pre-incubated with the vehicle (dimethylsulfoxide) or 10 μmol/L of antimycotics for 30 min and then incubated with 100 μg/mL *M. restricta* extract for 15 min. Results represent four separate experiments. ME, *M. restricta* extract; ITC, itraconazole; KTC, ketoconazole; FLC, fluconazole; VRC, voriconazole; LLC, luliconazole; TRB, terbinafine; BTN, butenafine; AMR, amorolfine.

a physical interaction between the antimycotics and the *M. restricta* extract may not be essential.

15d-PGJ_2 suppresses *M. restricta* extract-induced STAT1 activity and CXCL10 production

The cyclopentenone prostaglandin 15d-PGJ_2 inhibits the JAK/STAT pathway by suppressing the activation of STAT1, STAT3, JAK1 or JAK2.[16–18] Exogenous 15d-PGJ_2: (i) binds to and stimulates cell-surface PGD_2 receptors, the D prostanoid receptor (DP) or the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2); (ii) enters cells and binds to the intracellular receptor peroxisome proliferator-activated receptor (PPAR)-γ; or (iii) independent of these receptors, covalently interacts with JAK through the reactive electrophilic α,β-unsaturated carbonyl group within the cyclopentenone ring.[18–20] We examined if 15d-PGJ_2 suppressed *M. restricta* extract-induced STAT1 activity and CXCL10 production in a DP-, CRTH2- or PPAR-γ-dependent manner. 15d-PGJ_2 suppressed *M. restricta* extract-induced CXCL10 secretion (Fig. 6a), CXCL10 mRNA expression (Fig. 6b), STAT1 activity (Fig. 6c) and STAT1 phosphorylation (Fig. 6d). In contrast, *M. restricta* extract-induced CXCL10 secretion, CXCL10 mRNA expression, STAT1 activity and STAT1 phosphorylation were not suppressed by the other prostanoids, PGE_2 and $PGF_{2\alpha}$, or by the DP, CRTH2 and PPAR-γ agonists BW245C, 13,14-dihydro-15-keto-PGD_2 and rosiglitazone, respectively (Fig. 6a–d). These results indicate that DP, CRTH2 and PPAR-γ are not involved in the 15d-PGJ_2-induced suppression of CXCL10 production and STAT1 activity and that these responses are not suppressed by PGE_2 or $PGF_{2\alpha}$. 9,10-dihydro-15d-PGJ_2 (CAY10410), a structurally modified 15d-PGJ_2 lacking the α,β-unsaturated carbonyl (Fig. 6e) failed to suppress *M. restricta* extract-induced CXCL10 secretion, CXCL10 mRNA expression, STAT1 activity and STAT1 phosphorylation (Fig. 6a–d). The results indicate that the suppressive effects of 15d-PGJ_2 require the reactive α,β-unsaturated carbon within the cyclopentenone ring.

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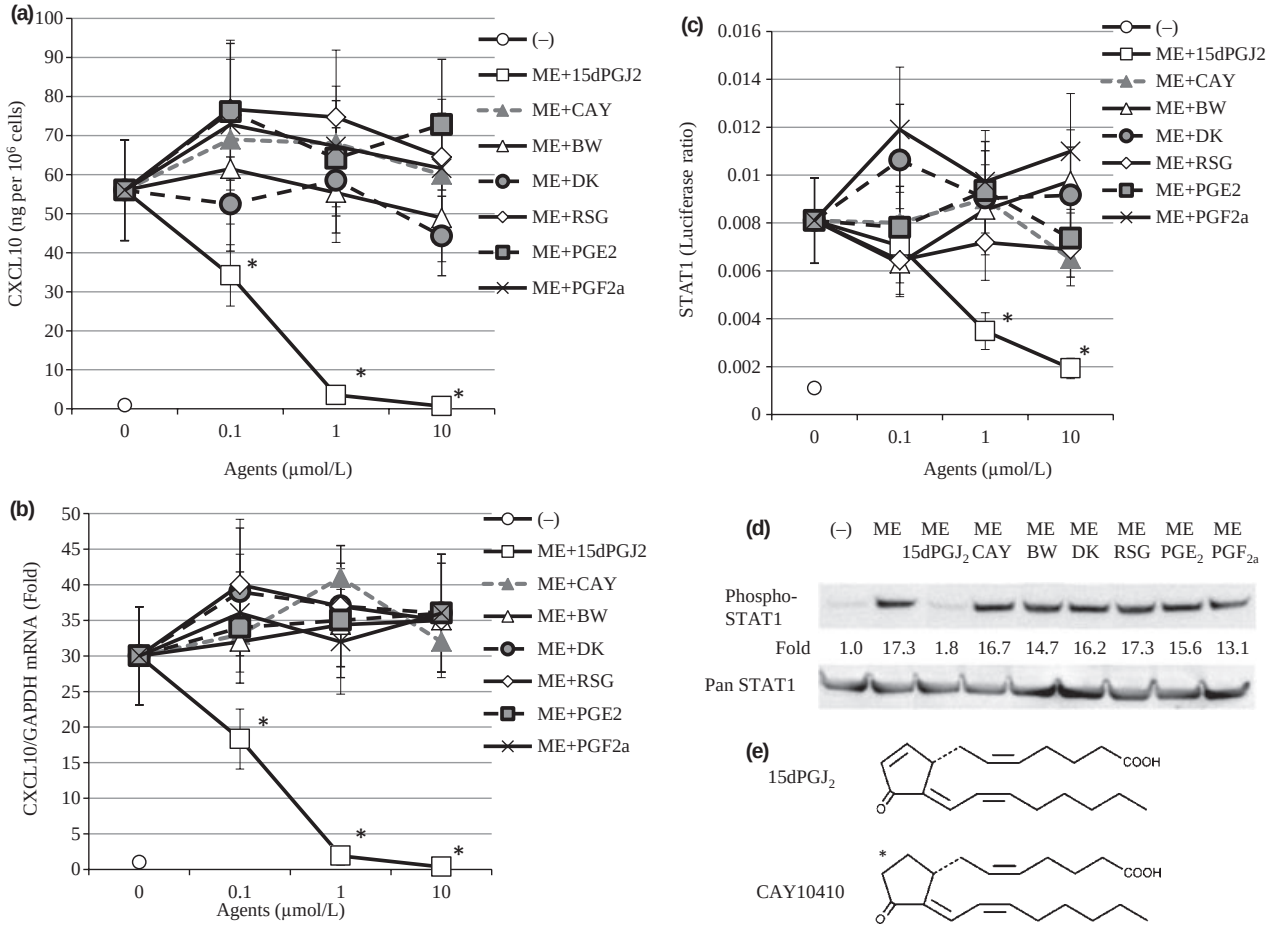

Figure 6. Effects of prostanoids on *Malassezia restricta* extract-induced CXC chemokine ligand (CXCL)10 secretion (a), CXCL10 mRNA expression (b), signal transducer and activator of transcription (STAT)1 activity (c) and STAT1 phosphorylation (d). (a–c) The keratinocytes, untransfected (a,b) or transfected with the STAT1-responsive firefly luciferase vector and *Renilla* luciferase vector (c), were pre-incubated with the vehicle (dimethylsulfoxide) or the indicated concentrations of 15-deoxy-$\Delta^{-12,14}$-prostaglandin J_2 (15d-PGJ_2), CAY10410 (CAY), BW245C (BW), 13,14-dihydro-15-keto-PGD_2 (DK), rosiglitazone (RSG), PGE_2, or $PGF_{2\alpha}$ for 30 min, followed by incubation with 100 μg/mL of *M. restricta* extract (ME). CXCL10 secretion, CXCL10 mRNA levels and STAT1 activity were analyzed at 48, 24 and 18 h, respectively. *$P < 0.05$ versus ME alone by one-way ANOVA with Dunnett's test. Mean ± standard deviation ($n = 4$) is shown. (d) The keratinocytes were pre-incubated with the vehicle (dimethylsulfoxide) or 10 μmol/L 15d-PGJ_2, CAY, BW, DK, RSG, PGE_2 or $PGF_{2\alpha}$ for 30 min, followed by incubation with 100 μg/mL ME for 15 min. The results represent four separate experiments. (e) The structure of 15d-PGJ_2 and CAY is shown.

Itraconazole, ketoconazole, luliconazole, terbinafine, butenafine and amorolfine increase the release of 15d-PGJ_2 and its precursor PGD_2 and decrease TXB_2 release in *M. restricta* extract-stimulated keratinocytes

15-Deoxy-$\Delta^{-12,14}$-prostaglandin J_2 is intracellularly produced from its precursor, PGD_2, by non-enzymatic dehydration. PGD_2 is intracellularly produced from PGH_2 by lipocalin-type PGD synthase (LPGDS) or hematopoietic PGD synthase (HPGDS).[19,20] PGH_2 is also converted to thromboxane A_2 (TXA_2) by thromboxane A synthase (TXAS) and to PGE_2 or $PGF_{2\alpha}$ by PGE_2 or $PGF_{2\alpha}$ synthases, respectively.[14] We recently found that itraconazole, ketoconazole, luliconazole, terbinafine, butenafine and amorolfine increase 15d-PGJ_2 release from keratinocytes in the presence of polyinosinic–polycytidylic acid plus IL-4.[10] We examined if these antimycotics also increased 15d-PGJ_2 release from keratinocytes treated with the *M. restricta* extract. 15d-PGJ_2 release was not altered by the *M. restricta* extract, but was increased by the addition of itraconazole, ketoconazole, luliconazole, terbinafine, butenafine and amorolfine (Fig. 7a). Fluconazole or voriconazole did not alter 15d-PGJ_2 release (Fig. 7a). Similarly, the release of PGD_2 was not altered by the *M. restricta* extract, but was increased by the addition of itraconazole, ketoconazole, luliconazole, terbinafine, butenafine and amorolfine (Fig. 7b). Fluconazole or voriconazole did not alter PGD_2 release (Fig. 7b). In contrast, *M. restricta* extract increased the release of TXB_2, a metabolite of TXA_2 (Fig. 7c),

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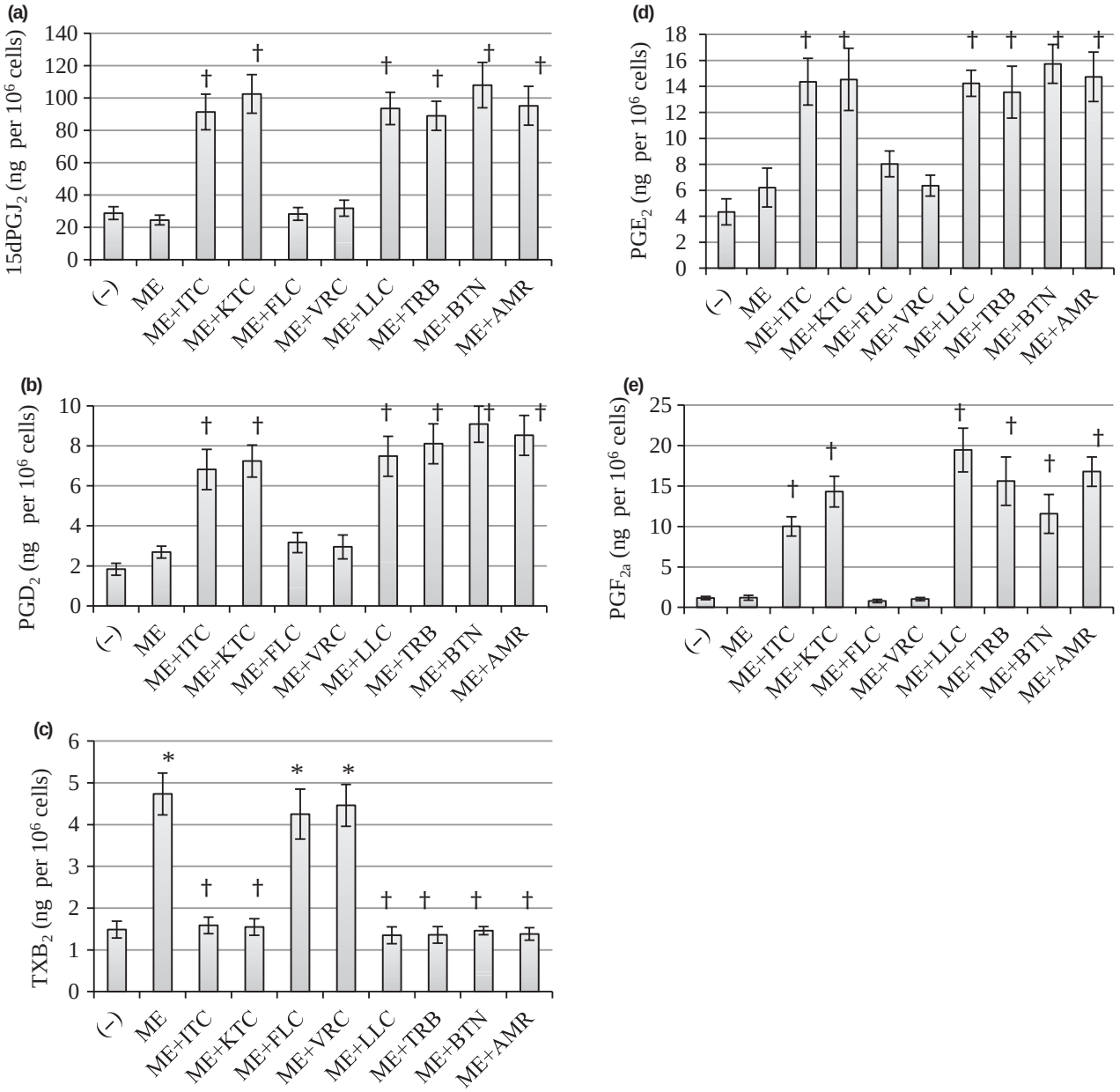

Figure 7. Effects of antimycotics on the release of 15-deoxy-$\Delta^{-12,14}$-prostaglandin J_2 (15d-PGJ_2) (a), PGD_2 (b), TXB_2 (c), PGE_2 (d) and $PGF_{2\alpha}$ (e) from *Malassezia restricta* extract (ME)-stimulated keratinocytes. Keratinocytes were pre-incubated with the vehicle (dimethylsulfoxide) or 10 μmol/L of antimycotics for 30 min, followed by incubation with 100 μg/mL ME extract for 48 h. The culture supernatants were analyzed for prostanoids. *$P < 0.05$ versus controls and †$P < 0.05$ versus ME alone by one-way ANOVA with Scheffé's test. Mean ± standard deviation ($n = 4$) is shown. ITC, itraconazole; KTC, ketoconazole; FLC, fluconazole; VRC, voriconazole; LLC, luliconazole; TRB, terbinafine; BTN, butenafine; AMR, amorolfine.

and this effect was reduced by itraconazole, ketoconazole, luliconazole, terbinafine, butenafine and amorolfine, but not by fluconazole or voriconazole (Fig. 7c). We also analyzed if antimycotics altered PGE_2 and $PGF_{2\alpha}$ release in the presence of the *M. restricta* extract. PGE_2 and $PGF_{2\alpha}$ release was not altered by the *M. restricta* extract, but was increased by itraconazole, ketoconazole, luliconazole, terbinafine, butenafine and amorolfine (Fig. 7d,e). Fluconazole or voriconazole did not alter PGE_2 and $PGF_{2\alpha}$ release (Fig. 7d,e). Thus, itraconazole, ketoconazole, luliconazole, terbinafine, butenafine and

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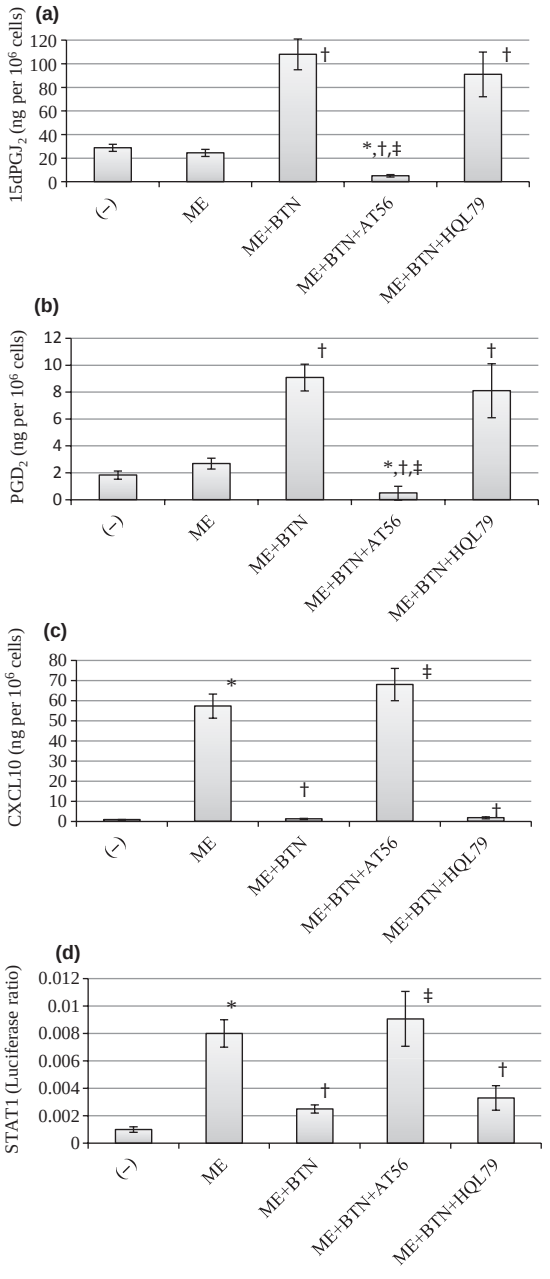

Figure 8. Butenafine cannot suppress CXC chemokine ligand (CXCL)10 secretion and signal transducer and activator of transcription (STAT)1 activity when 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) production is restricted. Keratinocytes, untransfected (a,c) or transfected with STAT1-responsive firefly luciferase vector and *Renilla* luciferase vector (d), were pre-incubated with the vehicle (dimethylsulfoxide) or 100 μmol/L AT-56 or HQL-79 for 30 min, incubated with 10 μmol/L butenafine (BTN) for 30 min, and then treated with 100 μg/mL *Malassezia restricta* extract (ME). The secretion of CXCL10 or prostanoids was analyzed at 48 h, and STAT1 activities were analyzed at 18 h. $*P < 0.05$ versus controls, $\dagger P < 0.05$ versus ME, and $\ddagger P < 0.05$ versus ME plus BTN by one-way ANOVA with Scheffé's test. Mean ± standard deviation ($n = 4$) is shown.

amorolfine may enhance the release of 15d-PGJ_2, PGD_2, PGE_2 and $PGF_{2\alpha}$, while suppressing TXB_2 release in *M. restricta* extract-stimulated keratinocytes.

Antimycotic-induced suppression of *M. restricta* extract-induced CXCL10 production and STAT1 activity may involve 15d-PGJ_2

We analyzed if 15d-PGJ_2 was involved in the antimycotic-induced suppression of CXCL10 production and STAT1 activity by using the LPGDS and HPGDS inhibitors AT-56 and HQL-79, respectively. AT-56 potently reduced 15d-PGJ_2 and PGD_2 release from cells treated with *M. restricta* extract plus butenafine to levels far below those of controls, whereas HQL-79 did not (Fig. 8a,b), indicating LPGDS-dependent production of PGD_2 and 15d-PGJ_2. In the presence of AT-56, when the production of 15d-PGJ_2 is highly restricted, butenafine did not suppress CXCL10 secretion (Fig. 8c), CXCL10 mRNA expression (data not shown) and STAT1 activity (Fig. 8d). Conversely, in the presence of HQL-79, when the production of 15d-PGJ_2 is sustained, butenafine did suppress CXCL10 secretion (Fig. 8c), CXCL10 mRNA expression (data not shown) and STAT1 activity (Fig. 8d). Similar results were obtained for 15d-PGJ_2, PGD_2, CXCL10 secretion, CXCL10 mRNA expression and STAT1 activity in cells treated with *M. restricta* extract plus itraconazole, ketoconazole, luliconazole, terbinafine or amorolfine (data not shown). The results suggest that the suppression of STAT1 activity and CXCL10 production induced by these antimycotics requires LPGDS-dependent production of 15d-PGJ_2.

DISCUSSION

Malassezia restricta extract induced CXCL10 production by activating STAT1 through LPA1/3 receptor engagement in keratinocytes. It is hypothesized that lysophosphatidic acid, a component of the *Malassezia* lipid bilayer, binds and activates the G-protein-coupled receptors (GPCR) LPA1 or LPA3 on keratinocytes. Ligand binding to several GPCR induces the JAK/STAT pathway in a $G_{i/o}$ or G_q protein-dependent or -independent manner.[21–23] LPA1 and LPA3 are coupled to the $G_{i/o,}$ $G_{12/13}$, or G_q proteins.[24] Therefore, it is hypothesized that lysophosphatidic acid from the *Malassezia* lipid layer binds LPA1 or LPA3 and activates JAK1 or JAK2 to phosphorylate STAT1 in a G-protein-dependent or -independent manner. Lysophosphatidic acid induces STAT3 phosphorylation in a JAK-dependent manner in human ovarian cancer cells.[25]

The antimycotics itraconazole, ketoconazole, luliconazole, terbinafine, butenafine and amorolfine suppressed the *M. restricta* extract-induced STAT1 activity and STAT1-dependent production of CXCL10 by increasing 15d-PGJ_2 production. The cyclopentenone ring of 15d-PGJ_2 covalently reacts with JAK1 or JAK2 and inhibits its ability to phosphorylate STAT1 or STAT3.[16–18] The α,β-unsaturated carbon within the cyclopentenone ring is required for this inhibition,[18] which is consistent with our present results (Fig. 6). Thus, the possible inhibition of JAK1 or JAK2 by 15d-PGJ_2 through the α,β-unsaturated

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carbon may mediate the antimycotic-induced inhibition of STAT1 activation and CXCL10 production.

Itraconazole, ketoconazole, luliconazole, terbinafine, butenafine and amorolfine decreased the release of TXB_2, a TXA_2 metabolite, and simultaneously increased the release of PGD_2 and its metabolite, 15d-PGJ_2, as well as the other prostanoids, $PGF_{2\alpha}$ and PGE_2, in keratinocytes stimulated with the *M. restricta* extract (Fig. 7). We recently showed that these antimycotics also increase the basal release of 15d-PGJ_2, PGD_2, $PGF_{2\alpha}$ and PGE_2 and reduce the release of TXB_2 in the absence of *M. restricta* extract, indicating that their original effects are independent of *Malassezia*.[10] These antimycotics may inhibit TXAS activity and redirect the metabolism of PGH_2 from TXA_2 to the other prostanoids, including PGD_2, which is further non-enzymatically dehydrated to become 15d-PGJ_2[26] and suppresses STAT1 activation. PGE_2 or $PGF_{2\alpha}$, though simultaneously increased by antimycotics, may not inhibit STAT1 activation (Fig. 6). Ketoconazole directly binds to TXAS and suppresses its activity.[27] In contrast, fluconazole and voriconazole did not alter the release of 15d-PGJ_2, PGD_2, TXB_2, PGE_2 or $PGF_{2\alpha}$ (Fig. 7), indicating that these antimycotics may not suppress the activity of TXAS. However, voriconazole is as potent as ketoconazole in suppressing the growth of *Malassezia*, fluconazole is less potent but still suppressive.[28,29] Thus, suppressing CXCL10 production, possibly via TXAS suppression in human keratinocytes, may not always correlate with *Malassezia* growth suppression via the suppression of the ergosterol biosynthetic enzyme C14*a*-lanosterol demethylase (a target of azole antimycotics) in *Malassezia*. The binding affinity of voriconazole and fluconazole to human TXAS may be much lower than that of ketoconazole. Although C14*a*-lanosterol demethylase and TXAS are both cytochrome P450 enzymes, the binding mechanisms to azole agents may differ: azoles bind to C14*a*-lanosterol demethylase primarily through the interaction of the azole nitrogen with the P450 heme iron of this enzyme.[30] Thus, fluconazole and voriconazole, as well as ketoconazole, can tightly bind fungal C14*a*-lanosterol demethylase through the nitrogen–heme bond.[30] In contrast, to obtain stoichiometric binding to TXAS, the interaction of azole side-chains with the very hydrophobic distal substrate-binding domain of TXAS is required, in addition to the proximal heme–nitrogen bond.[31,32] Because voriconazole and fluconazole have hydrophilic side-chains,[33] their binding to the distal hydrophobic substrate-binding domain of TXAS may be less potent than that of ketoconazole, which has a lipophilic phenylpiperazinyl ether side-chain.[34] Consequently, voriconazole and fluconazole may fail to inhibit TXAS.

The *M. restricta* extract induced CXCL10, a Th1-attracting chemokine, but did not induce CCL20, CCL22/17 or CCL27 to attract Th17, Th2 or Th22 cells, respectively. This suggests that *M. restricta* preferentially induces the infiltration of Th1 cells in AD lesions through CXCL10 and therefore contributes to the switch from an acute Th2 response to a chronic Th1 response.[35] The lack of Th2-attracting chemokine (CCL22/17) induction indicates that *Malassezia* may not act as an AD triggering factor to prime the Th2 response. Instead, it may act in the chronic phase to sustain or exacerbate inflammation. IFN-γ secreted from Th1 cells may act on epidermal keratinocytes and induce their apoptosis, leading to tissue damage;[12,36] it may also enhance their production of CXCL10, amplifying the infiltration of Th1 cells. The expression of IFN-γ-inducible and apoptosis-related genes is enhanced in chronic AD lesions.[36] Adult AD patients with head, neck and upper torso dermatitis patterns are more likely to react to *Malassezia* patch tests and show Th1-type cytokine profiles in the positive patch test lesions, indicating that the Th1-inducing effect of *Malassezia* occurs predominantly in the seborrheic region.[37] It is hypothesized that *Malassezia*-induced CXCL10 expression is involved in the development of AD in the seborrheic region, but not in the typical intertriginous AD region, which may possibly manifest as Th2-dominant inflammation. Antimycotics suppress *in vitro* CXCL10 production in keratinocytes at 1–10 μM, which is close to the peak serum concentrations obtained by the oral administration of 200 mg ketoconazole, 200 mg itraconazole or 250 mg terbinafine[38] and also to the peak SC concentration obtained by topical treatment with 1% terbinafine cream.[39] Our experimental conditions (0.4 mL of 100 μg/mL *Malassezia* extract in a 24-well plate, equal to 21 $\mu g/cm^2$ area and corresponding to $1–2 \times 10^7$ yeast cells/cm^2) mimic those used to produce experimental *Tinea versicolor* in rabbits, where the ear skin was inoculated with approximately 1×10^7 yeast cells/cm^2.[40] Thus, our present experimental setting may be analogous to that of cutaneous *Malassezia* infection treated with antimycotics. This suggests that the antimycotics administrated *in vivo* can suppress *Malassezia*-induced production of CXCL10 in epidermal keratinocytes at physiological concentrations. An antimycotic treatment may suppress the *M. restricta*-induced expression of CXCL10 through 15d-PGJ_2 in AD lesions, especially in seborrheic regions, and thus block the possible *M. restricta*-induced Th2/Th1 switch and the chronicity of eczema. Clinical studies should be conducted to determine if treatment with these antimycotics can suppress CXCL10 expression in AD lesions.

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