Brief report

Targeted inhibition of EPAS1-driven IL-31 production by a small-molecule compound

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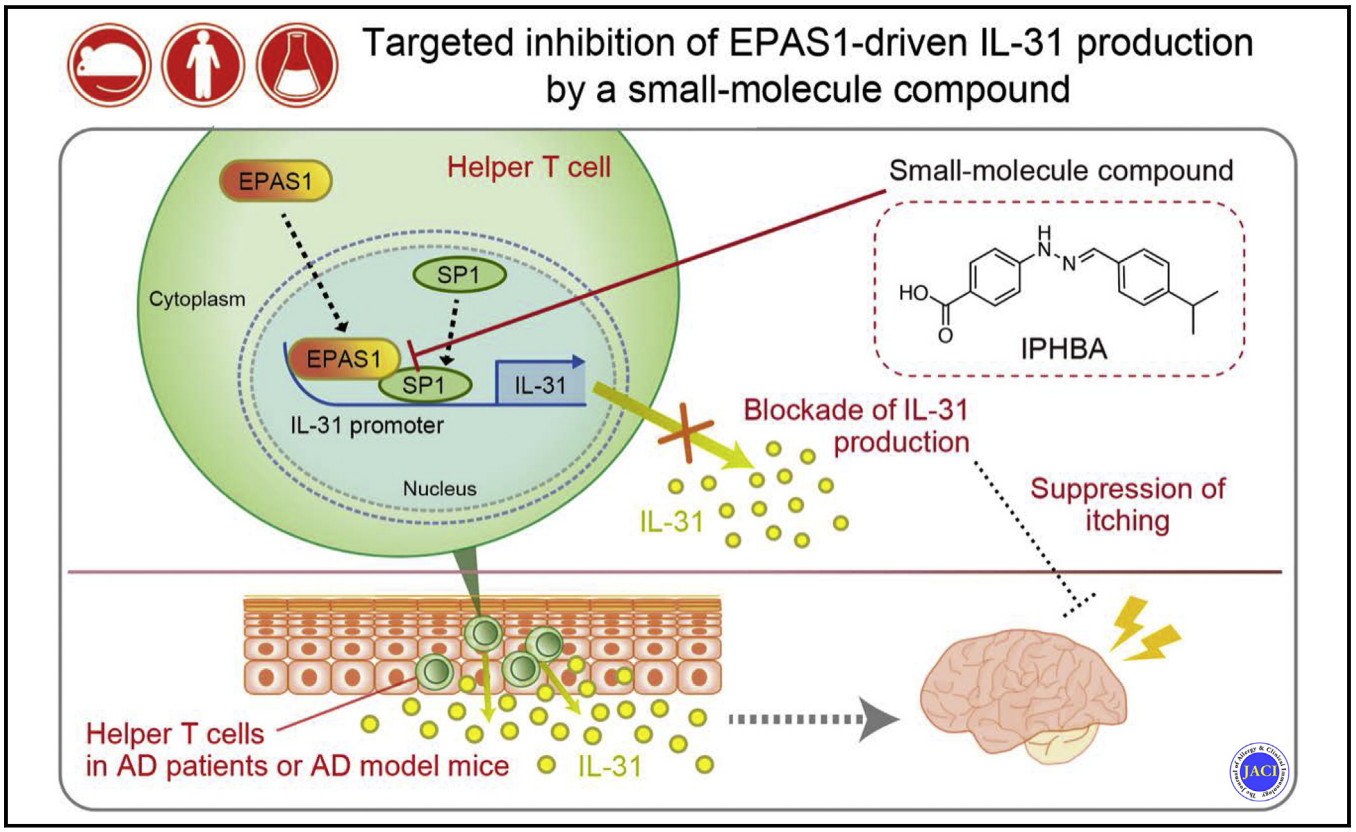
GRAPHICAL ABSTRACT



Background: IL-31 is a major pruritogen associated with atopic dermatitis (AD). Although a specific antibody for IL-31 receptor has been shown to alleviate pruritus in patients with AD, therapeutic approaches to inhibition of IL-31 production remain unexploited. IL-31 production by T_H cells critically depends on the transcription factor EPAS1, which mediates *IL31* promoter activation in collaboration with SP1.

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Objective: We aimed at developing small-molecule inhibitors that selectively block IL-31 production by T_H cells.
Methods: We generated the reporter cell line that inducibly expressed EPAS1 in the presence of doxycycline to mediate *Il31* promoter activation, and we screened 9600 chemical compounds. The selected compounds were further examined by using T_H cells from a spontaneous mouse model of AD and T_H cells from patients with AD.
Results: We have identified 4-(2-(4-isopropylbenzylidene) hydrazineyl)benzoic acid (IPHBA) as an inhibitor of *IL31* induction. Although IPHBA did not affect nonspecific T-cell proliferation, IPHBA inhibited antigen-induced IL-31 production by T_H cells from both an AD mouse model and patients with AD without affecting other cytokine production and hypoxic responses. In line with this, itch responses induced by adoptive transfer of IL-31–producing T_H cells were attenuated when mice were orally treated with IPHBA. Mechanistically, IPHBA inhibited the association between EPAS1 and SP1, resulting in defective recruitment of both transcription factors to the specific sites of the *IL31* promoter. We also determined the structure-activity relationship of IPHBA by synthesizing and analyzing 201 analogous compounds.
Conclusion: IPHBA could be a potential drug leading to inhibition of EPAS1-driven IL-31 production. (J Allergy Clin Immunol 2021;■■■:■■■-■■■.)

Key words: *Atopic dermatitis, IL-31, T_H cells, EPAS1, SP1, small-molecule compounds*

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by recurrent eczematous legions and intense itch.[1] Although many cytokines are involved in the pathogenesis of AD,[2] IL-31 plays a major role in AD-associated itch.[3-6] In patients with AD, IL-31 is mainly produced by $CD4^+$ T_H cells and transmits the signals via a heterodimeric receptor composed of an IL-31 receptor A and oncostatin M receptor.[3-7] A recent clinical study has demonstrated that blockade of IL-31 signals by a specific antibody for IL-31 receptor A alleviates pruritus in patients with AD.[8,9] However, therapeutic approaches to inhibition of IL-31 production by T_H cells remain unexploited.

Mutations of *DOCK8* in humans cause a combined immunodeficiency characterized by AD.[10,11] We have previously reported that DOCK8-deficient (*Dock8*$^{-/-}$) mice, but not *Dock8*$^{+/-}$ mice, spontaneously develop AD-like skin disease when crossed with transgenic mice expressing AND T-cell receptor (TCR) (designated Dock8$^{-/-}$ AND Tg mice).[12] Importantly, after stimulation with cognate antigen (moth cytochrome C peptide 88-103), $CD4^+$ T cells from *Dock8*$^{-/-}$ AND Tg mice produce large amounts of IL-31 in a manner dependent on the transcriptional factor EPAS1.[12] Although EPAS1 is known to control hypoxic response through the interaction with aryl hydrocarbon receptor nuclear translocator (ARNT),[13-15] EPAS1-mediated *IL31* promoter activation is independent of ARNT but occurs in collaboration with SP1.[12] Therefore, the EPAS1-SP1 axis might be a drug target for treatment of AD-associated itch.

Abbreviations used

AD:	Atopic dermatitis
ARNT:	Aryl hydrocarbon receptor nuclear translocator
Dock8$^{-/-}$ mice:	DOCK8-deficient mice
Dock8$^{-/-}$ AND Tg mice:	Dock8$^{-/-}$ mice expressing AND T-cell receptor
Dock8$^{-/-}$ OTII Tg mice:	Dock8$^{-/-}$ mice expressing OTII T-cell receptor
IPHBA:	4-(2-(4-Isopropylbenzylidene)hydrazineyl) benzoic acid
TCR:	T-cell receptor

RESULTS AND DISCUSSION

To identify small-molecule inhibitors of IL-31 production, we generated mouse embryonic fibroblasts, in which EPAS1 was inducibly expressed in the presence of doxycycline for mediation of *Il31* promoter activation (see Fig E1 in this article's Online Repository at www.jacionline.org). By experimentally testing 9600 compounds for their inhibitory activities, we found that 4 compounds (compounds **1**-**4**) inhibited antigen-induced gene expression of *Il31* when $CD4^+$ T cells from *Dock8*$^{-/-}$ AND Tg mice were treated with a concentration of 2.5 μM (Fig 1, *A* and *B*). One of these compounds is 4-(2-(4-isopropylbenzylidene)hydrazineyl)benzoic acid (IPHBA) (compound **2**). Although the other 3 compounds impaired nonspecific T-cell proliferation induced by phorbol 12–myristate 13–acetate plus ionomycin at a concentration of 20 μM (Fig 1, *C*), IPHBA did not show any adverse effects even when used at a concentration of 50 μM (see Fig E2 in this article's Online Repository at www.jacionline.org). Therefore, IPHBA was selected for further evaluation.

To examine the structure of IPHBA required for its inhibitory activity, we synthesized 201 IPHBA analogs. They include compound **5** (Fig 1, *A*), which has a *p*-(2-dimethylamino)ethoxy substituent instead of a *p*-isopropyl substituent on the right aromatic ring. Although treatment of $CD4^+$ T cells from *Dock8*$^{-/-}$ AND Tg mice with IPHBA at a concentration of 2.5 μM effectively inhibited antigen-induced *Il31* expression, no such inhibitory effect was seen for compound **5** (Fig 1, *D*). As the dimethylamino group is protonated under physiologic conditions to form a hydrophilic ammonium cation, these results suggest that the presence of a hydrophobic substituent at the *p* position on the right aromatic ring is necessary for biologic activity (see Fig E3 in this article's Online Repository at www.jacionline.org). On the other hand, IPHBA treatment did not affect the expression of *Il2* and *Il4* in these $CD4^+$ T cells (Fig 1, *D*). Similar results were obtained when $CD4^+$ T cells from *Dock8*$^{-/-}$ AND Tg mice were treated with IPHBA at a concentration of 20 μM and analyzed for production of IL-31 and IL-2 with ELISAs (Fig 1, *E*). Under this experimental setting, the half-maximal inhibitory concentration (IC_{50}) of IPHBA was 10.6 μM (see Fig E4 in this article's Online Repository at www.jacionline.org). Collectively, these results indicate that IPHBA selectively inhibits TCR-mediated IL-31 induction in murine $CD4^+$ T cells.

We found that IPHBA was readily detected for 12 hours in the blood of mice that had been orally treated at 100 mg/kg (see Fig E5 in this article's Online Repository at www.jacionline.org).

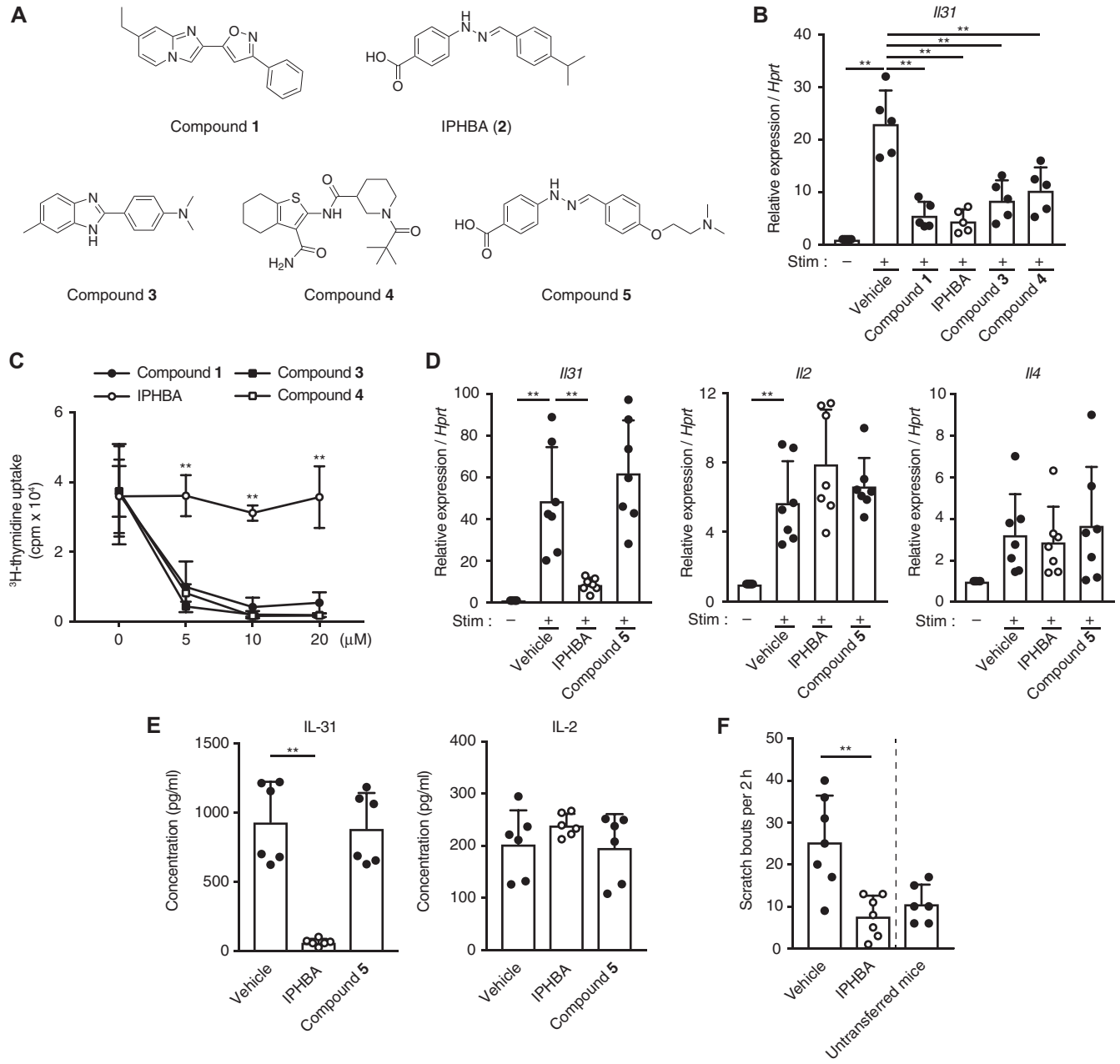

FIG 1. IPHBA selectively inhibits TCR-mediated *Il31* induction in murine CD4$^+$ T cells. **A,** Structure of the compounds used in this study. **B-E**, The effects of IPHBA on antigen-induced cytokine gene expression (**B** and **D**), phorbol 12–myristate 13–acetate plus ionomycin–induced proliferation (**C**), and antigen-induced cytokine production (**E**). **F,** The effect of IPHBA on scratching behavior of CAG-OVA mice induced by adoptive transfer of activated OTII CD4$^+$ T cells. Data are means ± SDs. $^{**}P < .01$ **B-E**, One-way ANOVA followed by the Dunnett *post hoc* test. **F**, Two-tailed unpaired Student *t* test. *Stim*, Stimulated.

This treatment did not affect body weight over 2 weeks (see Fig E6 in this article's Online Repository at www.jacionline.org), and the organs of the treated mice exhibited no macroscopic abnormalities at necropsy. To examine whether IPHBA treatment affects itch response, we used mice ubiquitously expressing ovalbumin under control of the cytomegalovirus immediate early enhancer–chicken β-actin hybrid promoter (designated CAG-OVA mice). As was the case for *Dock8*$^{-/-}$ AND Tg mice, CD4$^+$ T cells from *Dock8*$^{-/-}$ mice expressing OTII TCR (*Dock8*$^{-/-}$ OTII Tg mice) produced a large amount of IL-31 after stimulation with cognate peptide (OVA323-339).[12] When *in vitro*–activated CD4$^+$ T cells from *Dock8*$^{-/-}$ OTII Tg mice were adoptively transferred into CAG-OVA mice, scratching behavior was induced as compared with in untransferred mice (Fig 1, *F*). However, treatment of the transferred mice with IPHBA significantly suppressed scratching (Fig 1, *F*), indicating that orally administered IPHBA could function *in vivo*.

FM19G11 is not a direct inhibitor of EPAS1, but it suppresses *EPAS1* gene expression by acting on an undefined target.[16] When the human cancer cell line HT1080 was treated with FM19G11,

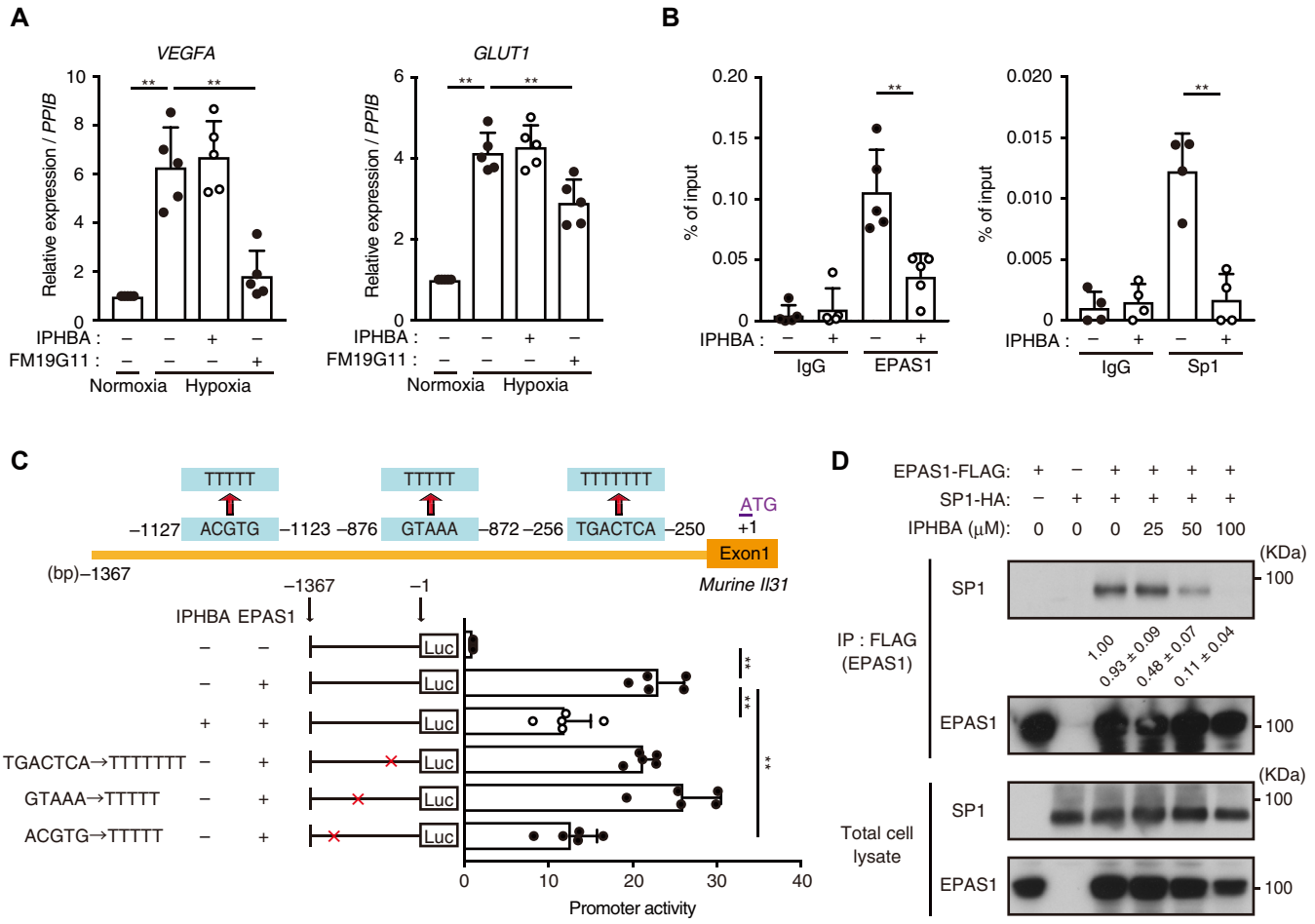

FIG 2. IPHBA inhibits the association between EPAS1 and SP1. **A**, The effect of IPHBA on hypoxic responses. **B**, Chromatin immunoprecipitation assay showing the effect of IPHBA on recruitment of EPAS1 and SP1 to the *Il31* promoter region. **C**, Identification of an EPAS1-binding site required for *Il31* promoter activation. **D**, Immunoprecipitation assays showing the effect of IPHBA on heterodimer formation between EPAS1 and SP1 (n = 3). Numerals indicate the ratio of associated SP1 to that of the control (vehicle alone). Data are means ± SDs. $^{**}P < .01$. **A** and **C**, One-way ANOVA followed by the Dunnett *post hoc* test. **B**, Two-tailed unpaired Student *t* test. *ATG*, Translation intiation codon; *HA*, Hemagglutinin; *IP*, immunoprecipitation; *Luc*, Luciferase.

hypoxia-induced expression of *VEGFA* and *GLUT1* was suppressed (Fig 2, *A*). In contrast, no such inhibitory effect was seen for IPHBA (Fig 2, *A*), suggesting that ARNT-dependent EPAS1 functions are unaffected by IPHBA.

To understand the mechanism through which IPHBA regulates IL-31 induction in $CD4^+$ T cells, we performed chromatin immunoprecipitation assays using $CD4^+$ T cells from *Dock8*$^{-/-}$ AND Tg mice. We found that after antigen stimulation, EPAS1 was recruited to the *Il31* promoter region (from –1336 to –1046) but its recruitment was significantly attenuated in the presence of IPHBA (Fig 2, *B*). This region includes a consensus EPAS1-binding sequence,[17] ACGTG, at the position from –1127 to –1123 (Fig 2, *C*). Indeed, mutation of ACGTG to TTTTT in the reporter construct diminished EPAS1-mediated *Il31* promoter activation (Fig 2, *C*), suggesting that this sequence is functionally important for EPAS1 binding. EPAS1 forms an "enhanceosome" through interaction with other transcription factors.[18] When FLAG-tagged EPAS1 was coexpressed with hemagglutinin-tagged SP1 in HEK-293Tcells, the association between EPAS1 and SP1 was readily detected (Fig 2, *D*). However, such heterodimer formation was impaired in the presence of IPHBA (Fig 2, *D*). Consistent with this, IPHBA treatment also attenuated SP1 recruitment to the specific site of *Il31* promoter (from –271 to +10) in $CD4^+$ T cells from *Dock8*$^{-/-}$ AND Tg mice (Fig 2, *B*). Thus, IPHBA inhibits the association between EPAS1 and SP1, resulting in defective recruitment of both transcription factors to the *Il31* promoter.

Having found that IPHBA selectively inhibits IL-31 induction in murine $CD4^+$ T cells, we next examined whether IPHBA also acts in human $CD4^+$ T cells. After stimulation with staphylococcal enterotoxin B, $CD4^+$ T cells from patients with AD produced larger amounts of IL-31 than did those from healthy controls (Fig 3, *A*). Importantly, IPHBA treatment again decreased the amount of IL-31 produced by human $CD4^+$ T cells without affecting IL-2 production (Fig 3, *A*). To better understand the effect of IPHBA on *IL31* promoter activation, we created a reporter construct containing an *IL31* promoter sequence (from –1308 to –1). When this reporter construct was expressed in mouse embryonic fibroblasts, *IL31* promoter activation was induced in the presence of human EPAS1 (Fig 3, *B*), which was significantly suppressed by IPHBA (Fig 3, *C*). By deleting the

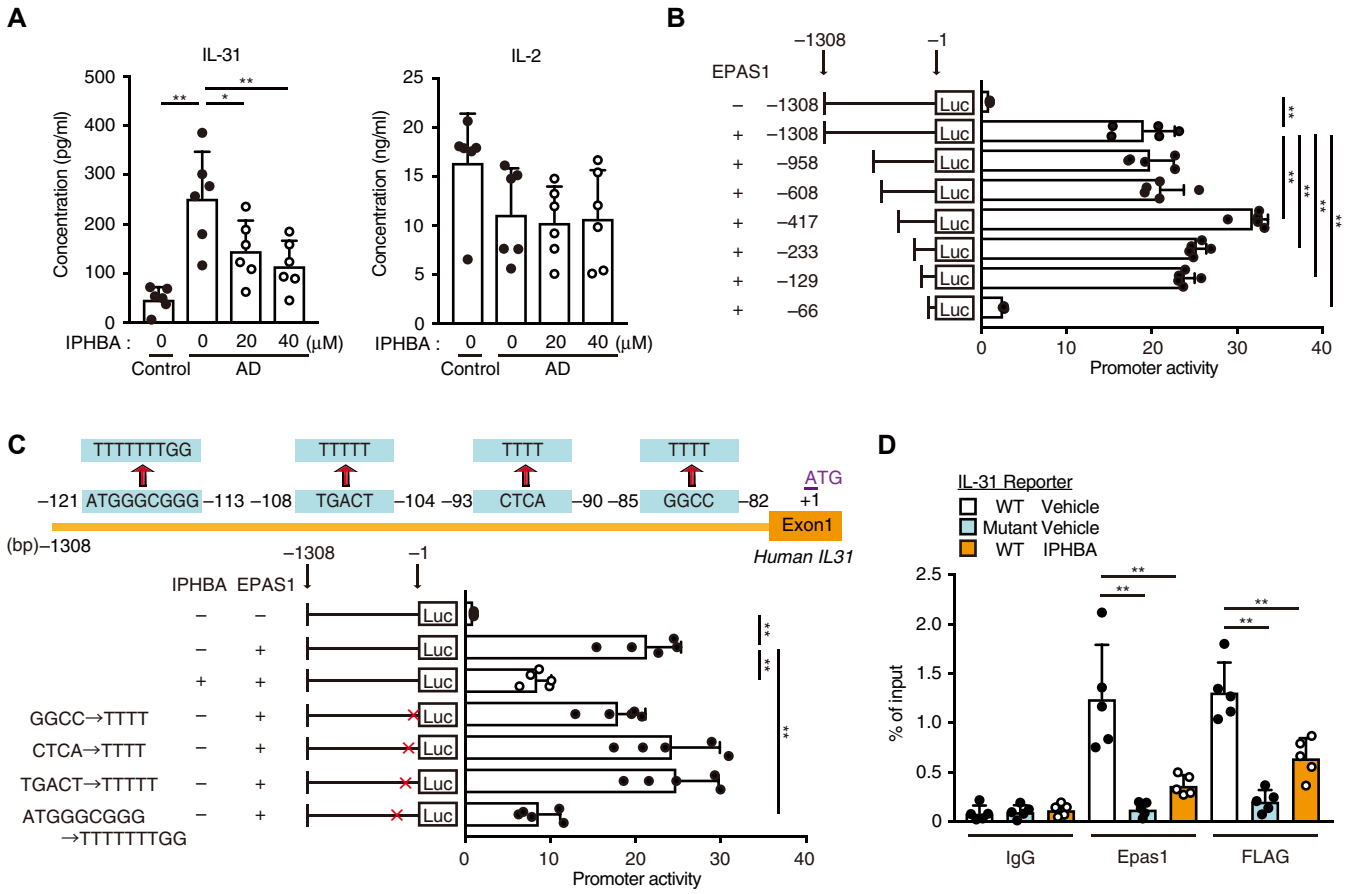

FIG 3. IPHBA inhibits IL-31 production by human CD4$^+$ T cells. **A**, The effect of IPHBA on staphylococcal enterotoxin B–induced cytokine production by CD4$^+$ T cells from patients with AD. **B** and **C**, Identification of the human *IL31* prompter region (**B**) and its sequence (**C**) critical for EPAS1-mediated transactivation. **D**, Chromatin immunoprecipitation assay showing that IPHBA inhibits recruitment of FLAG-tagged human EPAS1 to the *IL31* promoter region (from –1099 to –859). Data are means ± SDs. **P* < .05; ***P* < .01. **A**–**D**, One-way ANOVA followed by the Dunnett *post hoc* test. *ATG*, Translation initiation codon; *WT*, Wild-type; *Luc*, Luciferase.

IL31 promoter region, we identified the critical region for EPAS1-mediated transactivation (Fig 3, *B*), which included a consensus SP1-binding sequence,[19] TGGGCGGG, at the position from –120 to –113. When this sequence was mutated, EPAS1-mediated *IL31* promoter activation was diminished (Fig 3, *C*). Interestingly, disruption of SP1 binding also attenuated EPAS1 recruitment to the *IL31* promoter region (Fig 3, *D*). These results suggest that EPAS1 also forms a heterodimer with SP1 to mediate IL-31 induction in human CD4$^+$ T cells.

Here, we have identified IPHBA as a small-molecule inhibitor that suppresses EPAS1-driven IL-31 induction in murine and human CD4$^+$ T cells. Mechanistically, IPHBA inhibited the association between EPAS1 and SP1, resulting in defective recruitment of both transcription factors to the specific sites of *IL31* promoter. Although CD4$^+$ T cells are main source of IL-31 in AD,[3-6] other cells such as dermal dendritic cells also produce IL-31 in different contexts.[20] Therefore, how IPHBA affects IL-31 production by other cells is an important issue that should be investigated in future studies.

For treatment of AD, orally applicable small-molecule inhibitors of Janus kinase and phosphodiesterase 4 are currently undergoing development.[21,22] They are effective, but both inhibitors act on multiple cytokine pathways and their effects are nonspecific. In contrast, we have shown that IPHBA selectively inhibits *IL31* induction without affecting other cytokine production and hypoxic responses. Although the potency of IPHBA is not high enough, design and synthesis of IPHBA analogs with the electronic and structural properties identified in this study would lead to development of a novel drug for treatment of AD-associated itch.

Key messages

- **IPHBA was identified as a small-molecule inhibitor of IL-31 production.**
- **IPHBA inhibits IL-31 induction in murine and human T_H cells without affecting other cytokine production and hypoxic responses.**
- **IPHBA inhibits the association between EPAS1 and SP1 and impairs recruitment of both transcription factors to the specific sites of the *IL31* promoter.**

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