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Oclacitinib (APOQUEL®) is a novel Janus kinase inhibitor with activity against cytokines involved in allergy

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Janus kinase (JAK) enzymes are involved in cell signaling pathways activated by various cytokines dysregulated in allergy. The objective of this study was to determine whether the novel JAK inhibitor oclacitinib could reduce the activity of cytokines implicated in canine allergic skin disease. Using isolated enzyme systems and *in vitro* human or canine cell models, potency and selectivity of oclacitinib was determined against JAK family members and cytokines that trigger JAK activation in cells. Oclacitinib inhibited JAK family members by 50% at concentrations (IC_{50}'s) ranging from 10 to 99 nM and did not inhibit a panel of 38 non-JAK kinases (IC_{50}'s > 1000 nM). Oclacitinib was most potent at inhibiting JAK1 (IC_{50} = 10 nM). Oclacitinib also inhibited the function of JAK1-dependent cytokines involved in allergy and inflammation (IL-2, IL-4, IL-6, and IL-13) as well as pruritus (IL-31) at IC_{50}'s ranging from 36 to 249 nM. Oclacitinib had minimal effects on cytokines that did not activate the JAK1 enzyme in cells (erythropoietin, granulocyte/macrophage colony-stimulating factor, IL-12, IL-23; IC_{50}'s > 1000 nM). These results demonstrate that oclacitinib is a targeted therapy that selectively inhibits JAK1-dependent cytokines involved in allergy, inflammation, and pruritus and suggests these are the mechanisms by which oclacitinib effectively controls clinical signs associated with allergic skin disease in dogs.

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INTRODUCTION

Cytokines represent a class of secreted signaling proteins that function as chemical messengers that aid in cell-to-cell communication. These messengers can affect numerous cell behaviors such as growth, development, differentiation, and activation in a variety of immune cells as well as nonimmune cell types. Because of their role in regulating many important cellular functions, their dysregulation can orchestrate a variety of changes at the cellular and molecular level that translate into unwanted clinical signs and chronic disease conditions.

Cytokine dysregulation has been implicated in allergic skin disease, particularly atopic dermatitis (AD) in humans. The signature of cytokines that appear to be overproduced is T-helper type 2 (Th2) cytokines (e.g., IL-4, IL-5, IL-10, IL-13, IL-31), Th2-promoting cytokines (Thymic Stromal Lymphopoietin, IL-25, IL-33), and T-helper type 1 (Th1) cytokines (e.g., IFN-γ) (Ong & Leung, 2006; Brandt & Sivaprasad, 2011; Carmi-Levy *et al.*, 2011). Demonstrated involvement of these cytokines in the pathogenesis of human AD has triggered investigations into their role in canine AD. Work by several groups has shown that cytokine mRNA imbalance does exist in atopic dogs. Specifically, elevated levels of Th2 cytokines such as IL-4, IL-5, and IL-13 mRNA were seen in nonlesional and lesional atopic dog skin compared with skin from healthy control dogs. Additionally, IFN-γ and TNF-α transcripts were elevated in the skin of many atopic dogs, suggesting a mixed Th1-Th2 cytokine profile can be found, similar to what is seen in human AD (Olivry *et al.*, 1999; Nuttall *et al.*, 2002; Schlotter *et al.*, 2011). In an experimental model of canine allergic dermatitis, elevated cytokine transcripts of IFN-γ, IL-6, IL-13, and IL-18 were observed with atopy patch testing of house dust mite-sensitized, high IgE-producing beagles, further supporting the idea that cytokine dysregulation likely plays a role in allergic skin disease (Marsella *et al.*, 2006).

Janus kinase (JAK) enzymes, of which there are four family members (JAK1, JAK2, JAK3, and TYK2), play an important role in the activity of numerous cytokines. These enzymes reside in the cytoplasm of cells and selectively associate with

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the membrane proximal portion of a variety of type I and type II cytokine receptors. Upon cytokine stimulation of the receptor complexes, conformational changes in the cytoplasmic portion of the cytokine receptor occur, causing activation of receptor-associated JAKs. Activated JAK enzymes then phosphorylate intracellular domains of the cytokine receptor, creating docking sites for signaling proteins, notably, members of the signal transducers and activators of transcription (STAT) family. Once at the receptor, STATs are phosphorylated by JAKs on a conserved tyrosine residue. The STATs are then released from the receptor and dimerize with one another. These dimers translocate to the nucleus where they bind to specific DNA sequences and induce targeted gene transcription. Therefore, JAK enzymes play a key role in allowing extracellular proteins such as cytokines to transmit signals to the nucleus of target cells to initiate biological responses within the cell (Schindler & Plumlee, 2008; Harrison, 2012).

Numerous cytokines are known to activate the JAK family of enzymes when bound to their receptors. (Schindler & Plumlee, 2008) Cytokines implicated in allergic skin disease that utilize the JAK-STAT pathway include pro-inflammatory cytokines such as IL-2 and IL-6 as well as cytokines implicated in allergic responses (e.g., IL-4, IL-13) (Ong & Leung, 2006; Brandt & Sivaprasad, 2011). More recently, pruritogenic cytokines such as IL-31 have been shown to involve JAK-STAT activation when bound to their receptors (Dillon *et al.*, 2004; Gonzales *et al.*, 2013). Therefore, JAK inhibitors are thought to have utility in diseases such as allergic skin disease that involve dysregulated cytokine activity.

Oclacitinib (APOQUEL®; Zoetis Inc., Florham Park, NJ, USA) is a novel JAK inhibitor that has recently been approved in the United States and European Union for the control or treatment of pruritus associated with allergic dermatitis and the control or treatment of AD in dogs at least 12 months of age. The objective of this study was to evaluate the mechanism of action of oclacitinib by evaluating potency and selectivity toward JAK enzymes and to determine inhibitory activity toward cytokines involved in allergic skin disease.

MATERIALS AND METHODS

Investigational compound

Oclacitinib (Fig. 1) was synthesized in the laboratories of Zoetis (Kalamazoo, MI, USA).

Janus kinase enzyme activity assays and kinase selectivity panels

Recombinant human active kinase domains for JAK1 (amino acids 852–1142; NP_002218), JAK2 (amino acids 808–1132; NP_004963), JAK3 (amino acids 781–1124; NP_000206), and TYK2 (amino acids 870–1187; NP_003322) were used in isolated enzyme assays using Caliper microfluidics technology to determine potency of oclacitinib against the JAK family members, as previously described (Meyer *et al.*, 2010). Sequence homology to the analogous sequences in the canine

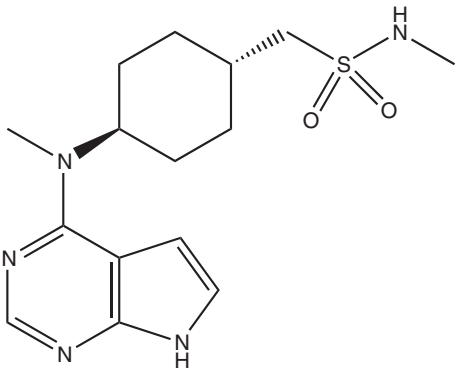

Fig. 1. Chemical structure of oclacitinib. Oclacitinib is a cyclohexylamino pyrrolopryimidine.

JAK enzymes are 98, 98, 100, and 90%, respectively (Figure S1). Invitrogen kinase panel testing (Life Technologies, Grand Island, NY, USA) was performed to determine potency of oclacitinib toward 38 different non-JAK kinases using their SelectScreen™ Kinase Profiling Services (Madison, WI, USA). Oclacitinib was evaluated at a concentration of 1 μM. Kinase-specific assay conditions and data analyses are described on their Web site http://www.lifetechnologies.com/us/en/home/products-and-services/services/custom-services/screening-and-profiling-services/selectscreen-profiling-service/selectscreen-kinase-profiling-service.html. This service utilizes the Z'-Lyte technology for all kinase screening. All tests were run in duplicate.

Interleukin-2 cytokine function

Canine whole blood was collected in heparin sulfate tubes from beagle dogs as well as dogs from mixed breeds. Whole blood (20 μL) was plated in 96-well plates (Corning Costar, Tewksbury, MA, USA) with 180 μL of DMEM complete medium (Dulbecco's modified Eagle medium; 10% heat-inactivated fetal bovine serum; 100 U/mL penicillin; 100 μg/mL streptomycin; Gibco Life Technologies, Grand Island, NY, USA) containing vehicle control or oclacitinib (0.001–10 μM), concanavalin A (ConA; 1 μg/mL; Sigma-Aldrich, St. Louis, MO, USA), and canine interleukin-2 (IL-2; 50 ng/mL; R&D Systems, Minneapolis, MN, USA). Plates were incubated at 37 °C for 48 h. Tritiated thymidine, 0.4 μCi per well (Perkin Elmer, Waltham, MA, USA), was added for 20 additional hours. Plates were frozen and then thawed, washed, and filtered using a Brandel MLR-96 cell harvester (Gaithersburg, MD, USA) and prewet filter mats (Perkin Elmer). Filters were dried and placed into filter sample bags (Perkin Elmer) with 10 mL of scintillant (Perkin Elmer). Sealed filters were counted on an LKB Wallac 1205 Betaplate liquid scintillation counter (Pharmacia, Uppsala, Sweden). Data were expressed as percent control, and dose–response data were then analyzed using a 4-parameter logistic equation.

Interleukin-4 cytokine function

The CellSensor® STAT6-*bla* RA-1 human B-cell line from Invitrogen (Life Technologies, Grand Island, NY, USA) was

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used. Cells were propagated in Opti-MEM Reduced Serum Medium containing 5% heat-inactivated fetal bovine serum, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 1% penicillin/streptomycin, and 556 ng/mL of CD40 Ligand (Invitrogen, Life Technologies) overnight at 37 °C, 5% CO_2. Cells were then plated in 384-well assay plates, black-wall, clear bottom (Costar Corning, Corning, NY, USA) at a density of 20 000 cells per well. Oclacitinib (0.0000381–10 μM) or vehicle control was added to cells for 2 h. Ten nanograms per milliliter hIL-4 (Invitrogen) was then added to cell cultures for 5 h. Activation of the STAT6-beta-lactamase reporter gene by IL-4 was determined by detecting beta-lactamase activity with the LiveBLAzer™-FRET B/G substrate (CCF-4 AM; Invitrogen) for 2 h. Fluorescence emission values at 460 and 530 nm were obtained using a fluorescent plate reader. The 460/530 nm ratios were expressed as percent control, and dose–response data were analyzed using a 4-parameter logistic equation.

Interleukin-6 cytokine function

The CellSensor® STAT3-*bla* HEK293T human epithelial cell line from Invitrogen was used. Cells were plated into 384-well assay plates, black-wall, clear bottom (Costar Corning) at a density of 1.875×10^5 cells per well in DMEM high glucose medium containing 5% FBS (Gibco Life Technologies) and incubated at 37 °C, 5% CO_2. Oclacitinib (0.0000954–25 μM) or vehicle control was added to cells for 1 h. Twenty nanograms per milliliter hIL-6 (Invitrogen) was then added to cell cultures for 5 h. Activation of the STAT3-beta-lactamase reporter gene by IL-6 was determined by detecting beta-lactamase activity with the LiveBLAzer™-FRET B/G substrate (CCF-4 AM; Invitrogen). Fluorescence emission values at 460 and 530 nm were obtained using a fluorescent plate reader. The 460/530 nm ratios were expressed as percent control, and dose–response data were analyzed using a 4-parameter logistic equation.

Interleukin-13 cytokine function

The HT-29 human colonic epithelial cell line from American Type Culture Collection (ATCC, Manassas, VA, USA) was used. Cells were propagated in McCoy's 5A medium containing 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamine (Gibco Life Techologies). Cells were trypsinized from flasks, washed in fresh medium, and resuspended in 96-well assay plates (Costar Corning) at a density of 3×10^5 cells per well. Oclacitinib (0.0015–30 μM)) or vehicle control was added to cells while on ice for 30 min. One nanogram per milliliter hIL-13 (R&D Systems) was then added. Cells were incubated in a 37 °C water bath for 30 min then fixed in 1.75% formaldehyde in PBS, washed in PBS containing 0.5% BSA, and incubated overnight at −20 °C in absolute methanol. Fixed cells were stained with PE-labeled antibody to human pSTAT6 (pY641; BD Biosciences, San Jose, CA, USA). Samples were analyzed with an FACSCalibur equipped with a plate-based autosampler (BD Biosciences) and analyzed using FlowJo software, version 7.6.1 (Tree Star Inc., Ashland, OR, USA). Data were expressed as mean fluorescence and then expressed as percent control. Dose–response data were then analyzed using a 4-parameter logistic equation.

Interleukin-31 cytokine function

The canine monocytic cell line DH82 (ATCC) was plated at a density of 100 000 cells per well in 96-well plates and grown overnight in MEM medium (Gibco Life Technologies) containing 15% FBS (Gibco Life Technologies) and 10 ng/mL canine IFNγ (R&D Systems). After 24 h, growth medium was replaced with serum-free MEM for 2 h. Test compound or vehicle control was then added for 1 h to generate an 11-point titration curve (ranging from 0.0001 to 10 μM). Cells were treated with 1 μg/mL of canine IL-31 (in-house generated) for 5 min. Cytokine treatment was terminated by removing medium and adding AlphaScreen SureFire™ lysis buffer (Perkin Elmer). Activation of pSTAT3 was detected using Perkin Elmer AlphaScreen SureFire™ STAT3 p-Y705 kit. Data were expressed as mean Alpha Screen Signal units and then expressed as percent control. Dose–response data were then analyzed using a 4-parameter logistic equation.

Granulocyte/macrophage colony-stimulating factor cytokine function

The Cell Sensor® irf-1-*bla* TF-1 human erythroleukemia cell line from Invitrogen was used. Cells were plated in 96-well plates (Costar Corning) at a density of 2×10^6 cells per well in Opti-MEM Reduced Serum Medium (Invitrogen) containing 5% heat-inactivated fetal bovine serum, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 1% penicillin/streptomycin (all from Gibco Life Technologies) and incubated at 37 °C, 5% CO_2. Oclacitinib (0.00061–10 μM) or vehicle control was added to cell cultures for 2 h. Cells were then stimulated with 10 ng/mL GM-CSF (Invitrogen) for 5 h. Activation of the STAT5-beta-lactamase reporter gene by GM-CSF-1 was determined by detecting beta-lactamase activity with the LiveBLAzer™-FRET B/G substrate (CCF-4 AM; Invitrogen) using a fluorescent plate reader. Fluorescence emission values at 460 and 530 nm were obtained using a fluorescent plate reader. The 460/530 nm ratios were expressed as percent control, and dose–response data were analyzed using a 4-parameter logistic equation.

Erythropoietin cytokine function

Canine bone marrow cells were isolated under sterile conditions from the humerus of beagle dogs and placed in warmed DMEM medium (Gibco Life Technologies). The sample was filtered through a stainless steel wire mesh (Sigma-Aldrich) and gently ground with a glass pestle. The suspension was then passed through a 100-μm cell strainer and washed three times in warmed Alsever's solution (Sigma-Aldrich). The final pellet was resuspended in 20 mL of DMEM complete medium. Cell

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suspensions were plated in 96-well plates (Costar Corning) at a density of 2×10^5 cells per well in DMEM complete medium containing oclacitinib (0.001–10 μM) or vehicle control, and erythropoietin (0.2 U per well Erythropoietin; R&D Systems). Plates were incubated at 37 °C for 48 h. Tritiated thymidine, 0.4 μCi per well (Perkin Elmer), was added for 20 additional hours. Plates were frozen and then thawed, washed, and filtered using a Brandel MLR-96 cell harvester and prewet filter mats (Perkin Elmer). Filters were dried at 60 °C for 1 h and placed into filter sample bags (Perkin Elmer) with 10 mL of scintillant (Perkin Elmer). Sealed filters were counted on an LKB Wallac 1205 Betaplate liquid scintillation counter. Data were expressed as percent control, and dose–response data were then analyzed using a 4-parameter logistic equation.

Interleukin-12 cytokine function

Human whole blood was collected in heparin sulfate tubes. Whole blood (75 μL per well) was plated in 384-well plates (Costar Corning) containing vehicle control or oclacitinib (0.000143–37.5 μM). Treated whole blood was then stimulated with mouse anti-human CD3 (1 μg/mL; BD Biosciences), mouse anti-human CD28 (10 ng/mL; BD Biosciences), and recombinant human IL-12 (10 ng/mL; R&D Systems) overnight at 37 °C, 5% CO_2. Whole blood was spun to separate plasma, and 20 μL of plasma was collected. Interferon gamma levels were quantitated in plasma using Meso IFN-gamma 384-well plate kit and a Meso Scale Discovery SECTOR Imager 6000 (Rockville, MD, USA) following the manufacturer's instructions. Data were expressed as percent control, and dose–response data were then analyzed using a 4-parameter logistic equation.

Interleukin-23 cytokine function

Human whole blood was collected in heparin sulfate tubes. Whole blood (70 μL) was plated in 96-well, deep-well, V-bottom plates (Corning) and incubated at 37 °C for 30 min. Vehicle control or oclacitinib (0.000143–37.5 μM) was added to plates and incubated at 37 °C for 30 min. IL-23 (100 ng/mL; R&D Systems) was then added to plates and incubated at 37 °C for 15 min. Reactions were stopped by the addition of 1× Phosflow lysis/fix buffer (BD Biosciences). Plates were incubated at RT for 15 min. Cells were washed in PBS buffer containing 1% heat-inactivated fetal bovine serum (Gibco Life Technologies) and 0.01% sodium azide and permeablized in 90% ice cold methanol for 30 min. Cells washed and stained with AlexaFluor 647-conjugated anti-pSTAT3 antibody (BD Biosciences) diluted 1:150. Plates were incubated at 4 °C overnight. Samples were transferred to 96-well U-bottom plates (Costar Corning) for flow cytometric analysis. Flow cytometric analysis was performed using a FACSCalibur flow cytometer equipped with the BD High Throughput sampler using CellQuest software (BD Biosciences). Thirty thousand cellular events were collected per sample, and data were analyzed using FlowJo software (Tree star). Lymphocyte populations showing pSTAT3 staining were enumerated across dose groups. Data were expressed as percent control, and dose–response data were then analyzed using a 4-parameter logistic equation.

RESULTS

Inhibitory activity of oclacitinib against JAK family members was determined in isolated enzyme systems. Oclacitinib inhibited JAK1, JAK2, JAK3, and TYK2 by 50% at concentrations (IC_{50}'s) of 10, 18, 99, and 84 nM, respectively (Table 1). Oclacitinib was most potent against the JAK1 enzyme, showing a 1.8-fold selectivity for JAK1 vs. JAK2 and 9.9-fold selectivity toward JAK1 vs. JAK3 (Table 1). Oclacitinib did not significantly inhibit a panel of 38 other kinases when tested at a concentration of 1 μM (Table 2).

Effects of oclacitinib on the function of cytokines involved in inflammation (IL-2 and IL-6), allergy (IL-4 and IL-13), pruritus (IL-31) as well as hematopoiesis (erythropoietin and GM-CSF), and innate immune cell responses (IL-12 and IL-23) were specifically evaluated. Oclacitinib was able to inhibit IL-2, IL-4, IL-6, IL-13, and IL-31 with IC_{50}'s ranging from 36 to 249 nM. Oclacitinib was much less potent against erythropoietin and GM-CSF, with IC_{50}'s above 1000 nM for both of these cytokines. Oclacitinib did not significantly inhibit IL-12 or IL-23 function (IC_{50}'s > 3000 nM) (Table 3).

DISCUSSION

Janus kinase inhibitors are being developed by numerous pharmaceutical companies for the treatment of a variety of inflammatory, autoimmune, and hematologic disorders in humans. The selectivity profile toward JAK family members varies between these different inhibitors, and desired profiles are influenced in part by the disease indication of interest (Menet *et al.*, 2013). A variety of cytokines implicated in allergy require activation of a cytokine receptor that utilizes JAK1 as one of the obligate JAK partners (Fig. 2). Therefore, a JAK1 selective inhibitor may be useful for the treatment of allergic conditions in dogs. In this study, we characterized the *in vitro* activity of the novel JAK inhibitor oclacitinib and showed it preferentially inhibited JAK1-dependent cytokine receptor complexes involved

Table 1. Inhibitory activity of oclacitinib against Janus kinase (JAK) family members in isolated enzyme systems

Assay	Mean IC_{50} (nM) ± SE†
JAK1 isolated enzyme*	10 ± 1
JAK2 isolated enzyme*	18 ± 2
JAK3 isolated enzyme*	99 ± 20
TYK2 isolated enzyme*	84 ± 12

*Human recombinant enzymes used in Caliper enzyme assay format. ATP conc used = K_m of enzyme.

†Arithmetic means representing the concentration of oclacitinib that inhibits activity by 50% (IC_{50}) ± SE are presented (n = 3).

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Table 2. Invitrogen kinase screening data

Kinase	Percent inhibition at 1000 nM oclacitinib (1st replicate)	Percent inhibition at 1000 nM oclacitinib (2nd replicate)
ABL tyrosine kinase	13	13
RAC-alpha serine/threonine kinase (Protein kinase B)	3	0
ZAP-70 tyrosine kinase	12	9
Angiopoietin 1 receptor (TIE-2) (TEK)	3	−1
Aurora-related kinase 1 (ARK1)	40	40
Ste20-like kinase MST2	22	14
SRC kinase	19	15
Serum glucocorticoid-regulated kinase (SGK) (SGK1)	16	11
p160ROCK protein kinase	34	32
Protein kinase C beta II Isoform	8	5
Protein kinase A alpha	19	14
Serine/threonine protein kinase PIM-2	13	6
PAK-4 (p21 activated kinase 4)	18	9
High affinity nerve growth factor receptor (TRK-A)	32	31
Serine/threonine protein kinase NEK2	4	3
Myosin light chain kinase (skeletal)	12	−1
Serine/threonine protein kinase MASK	6	3
Hepatocyte growth factor receptor (MET Proto-oncogene tyrosine kinase)	21	9
Serine/threonine protein kinase MARK	43	40
MAP kinase activated protein kinase 2 (MAPKAPK-2)	6	0
Mitogen-activated protein kinase 1 (MAPK1/ERK2)	10	6
Mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4/HGK)	42	34
Proto-oncogene LCK tyrosine kinase	22	22
Vascular endothelial growth factor receptor 2 (VEGFR-2/FLK1)	41	37
Tyrosine-protein kinase JAK3 (JANUS KINASE 3)	98	92
Insulin receptor	7	4
Glycogen synthase kinase-3 beta (GSK-3 beta)	9	8
FGFR1 FGF receptor 1	12	8
Ephrin type-A receptor 2 (ECK)	9	2
Epidermal growth factor receptor	10	6
Casein kinase II alpha prime chain (CK II)	5	3
Casein kinase 1, alpha 1	1	1
Checkpoint kinase (CHK2)	9	5
Checkpoint kinase CHK1	21	6
CDK2/Cyclin-A	11	10
Calcium/calmodulin-dependent protein kinase II alpha-B subunit	22	15
Bruton's tyrosine kinase (BTK)	8	6
TAO kinase 2	20	-2
Mitogen-activated protein kinase 14 (MAPK14/P38-alpha)	9	7

in inflammation and pruritus, common clinical signs in canine allergic skin disease.

In isolated enzyme systems, oclacitinib was shown to inhibit JAK family members, most effectively JAK1, while not significantly inhibiting a variety of other non-JAK kinases, suggesting oclacitinib is a selective JAK inhibitor. Exploration of oclacitinib's selectivity pattern in cell model systems showed that oclacitinib preferentially inhibited pro-allergic, pro-inflammatory, and pruritogenic cytokines that bind receptors utilizing the JAK1 enzyme as one of the JAK partners to mediate intracellular signaling. For example, oclacitinib inhibited IL-2 and IL-4 that bind receptor complexes that engage both JAK1 and JAK3 enzymes to trigger signal transduction cascades. IL-6, IL-13, and IL-31 were also inhibited by oclacitinib. These cytokines bind receptor complexes that activate JAK1 and JAK2 enzymes and possibly TYK2 under some circumstances (Schindler & Plumlee, 2008) (Fig. 2). Oclacitinib inhibited these JAK1-dependent cytokines with IC_{50}'s ranging from 36 to 249 nM.

The JAK1-dependent cytokines evaluated in this study play a role in many aspects of allergy. IL-4 is a cytokine produced by Th2 cells, mast cells, and eosinophils. It functions to regulate immunoglobulin class switching in B cells and enhances the expression of FcεRI, the high affinity receptor that binds

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Table 3. Inhibitory activity of oclacitinib in cell model systems

Assay	Mean IC_{50} (nM) ± SEM*
Canine IL-2 function in beagle whole blood	63 ± 6
Canine IL-2 function in mixed breed whole blood	189 ± 39
Human IL-4 function in cells	249 ± 19
Human IL-6 function in cells	159 ± 58
Human IL-13 function in cells	113 ± 7
Canine IL-31 function in cells	36 ± 6
Canine (beagle) erythropoietin function in cells	1020 ± 189
Human GM-CSF function in cells	1090 ± 110
Human IL-12 function in cells	>3000
Human IL-23 function in cells	>3000

*Arithmetic means representing the concentration of oclacitinib that inhibits activity by 50% (IC_{50}) ± SEM (n = 2–24).

allergen-specific IgE on a variety of immune cells. IL-4 can also induce proliferation, survival, and/or chemotaxis of many cell types such as B cells, mast cells, and eosinophils, some of the key players in allergy (Izuhara *et al.*, 2002). IL-13 is also produced by Th2 cells, and receptors for this cytokine have been found on epithelial cells and macrophages. Its function has been studied more from the perspective of allergic airway disease, so the understanding of its role in allergic skin disease is limited (Wills-Karp & Finkelman, 2008). IL-6 is a cytokine produced by a variety of cell types including T cells and macrophages and plays a key role in activating the innate immune system (e.g., neutrophil migration to inflamed sites) and promoting B-cell differentiation and antibody production (Mihara *et al.*, 2012). IL-2 regulates T-cell proliferation and can induce the production of T-cell cytokines such as IFN-γ. Both IL-2 and IFN-γ transcripts have been found to be upregulated in the skin of atopic dogs (Nuttall *et al.*, 2002), suggesting that IL-2 can contribute to cellular and molecular changes that drive inflammation in the skin. Finally, IL-31 is a recently identified cytokine that can induce scratching behavior in dogs when injected into the animal and has also been found in dogs with naturally occurring AD (Gonzales *et al.*, 2013), suggesting IL-31 may be an important cytokine driving pruritic responses in dogs. The ability to inhibit JAK1-dependent cytokines discussed above is a desirable trait to have for a treatment of allergic skin disease where chronic inflammation and pruritus are important clinical signs to control in the animal.

Oclacitinib appears to have specificity toward cytokines involved in allergy, inflammation, and pruritus but shows reduced activity toward cytokines involved in hematopoiesis (GM-CSF and erythropoietin; IC_{50}'s > 1000 nM). Many hematopoietic cytokines bind homodimeric receptors that do not recruit JAK1 but instead recruit JAK2/JAK2 pairs for signal transduction and induction of proliferation of bone marrow cells (Schindler & Plumlee, 2008) (Fig. 2). Although oclacitinib appeared to be potent against JAK2 in isolated enzyme assays, potency toward cytokines that engage receptor complexes utilizing JAK2/JAK2 pairings was not significant in cell-based assays. The isolated enzyme reactions are contrived systems where the enzymes are artificially activated, and truncated versions of the enzyme are used in tests instead of full-length enzymes in their natural state, associated with their appropriate receptor within a cell, and activated by their natural ligand. Homology between the human and canine JAK2 amino acid sequences 808–1132, the sequence of the truncated JAK2 protein used in isolated enzyme reactions, is 98%, whereas homology between the human and canine full-length JAK2 protein is 94%. Although not significantly different, it is possible that we could not engineer the JAK2 enzyme to completely mimic its natural state within the cell, which could cause data generated from enzyme assays to be dramatically different from cell-based data. A similar disconnect in potency between the IC_{50} generated in JAK2 isolated enzyme assays and JAK2-dependent cell-based assays has also been noted with tofacitinib where the IC_{50} against JAK2 in the enzyme assay was reported to be 4.1 nM, but the IC_{50} was 1377 nM in whole-blood assays evaluating the activity of GM-CSF on human monocytic cells (Meyer *et al.*, 2010). As oclacitinib was less potent at inhibiting the function of cytokine receptor complexes utilizing JAK2/JAK2 pairings compared with the cytokines that could activate receptors that engaged JAK1/JAK3, JAK1/JAK2, or JAK1/JAK2/TYK2 pairings, these data suggest inhibition of JAK1 is driving the selectivity pattern of oclacitinib in cells.

Oclacitinib showed no activity toward the function of IL-12 and IL-23 (IC_{50}'s > 3000 nM) in human cell-based assays. IL-12 and IL-23 bind receptors that recruit JAK2 and TYK2 reconfirming minimal or no activity toward cytokine-receptor complexes that are not dependent on the JAK1 enzyme (Fig. 2). These cytokines are produced by antigen-presenting cells in response to microbial pathogens such as viruses, bacteria, and parasites and play a key role in engaging the adaptive immune system. Their activity is essential in preventing serious infections and inducing appropriate responses to fight off foreign invaders (Vignali & Kuchroo, 2012). Expanding our panel of cytokine testing to other JAK-dependent cytokines involved in similar innate immune cell activities such as type I interferons (IFN $\beta/\omega/\tau$) and type II IFN-γ will be important to better understand oclacitinib's effects on cytokines involved in innate immune cell responses.

As some of our cell model systems used to test the potency and selectivity of oclacitinib were human cell model systems, particularly those cytokine-receptor complexes that utilized the JAK2/TYK2 pairings (e.g., IL-12 and IL-23 functional cell-based tests), it is important to keep in mind the homology between the human and canine JAK sequences. The sequence homology between human and canine full-length JAK1, JAK2, JAK3, and TYK2 proteins are 98%, 94%, 100%, and 83%, respectively (Figure S1). Oclacitinib did not inhibit IL-12 and IL-23 function in human cell-based assays; however, these cytokines activate receptors that utilize JAK2 and TYK2 pairings. Human TYK2 is only 83% homologous to canine TYK2, which is much lower than the other JAK family members. Therefore, it is important to continue investigations of oclaciti-

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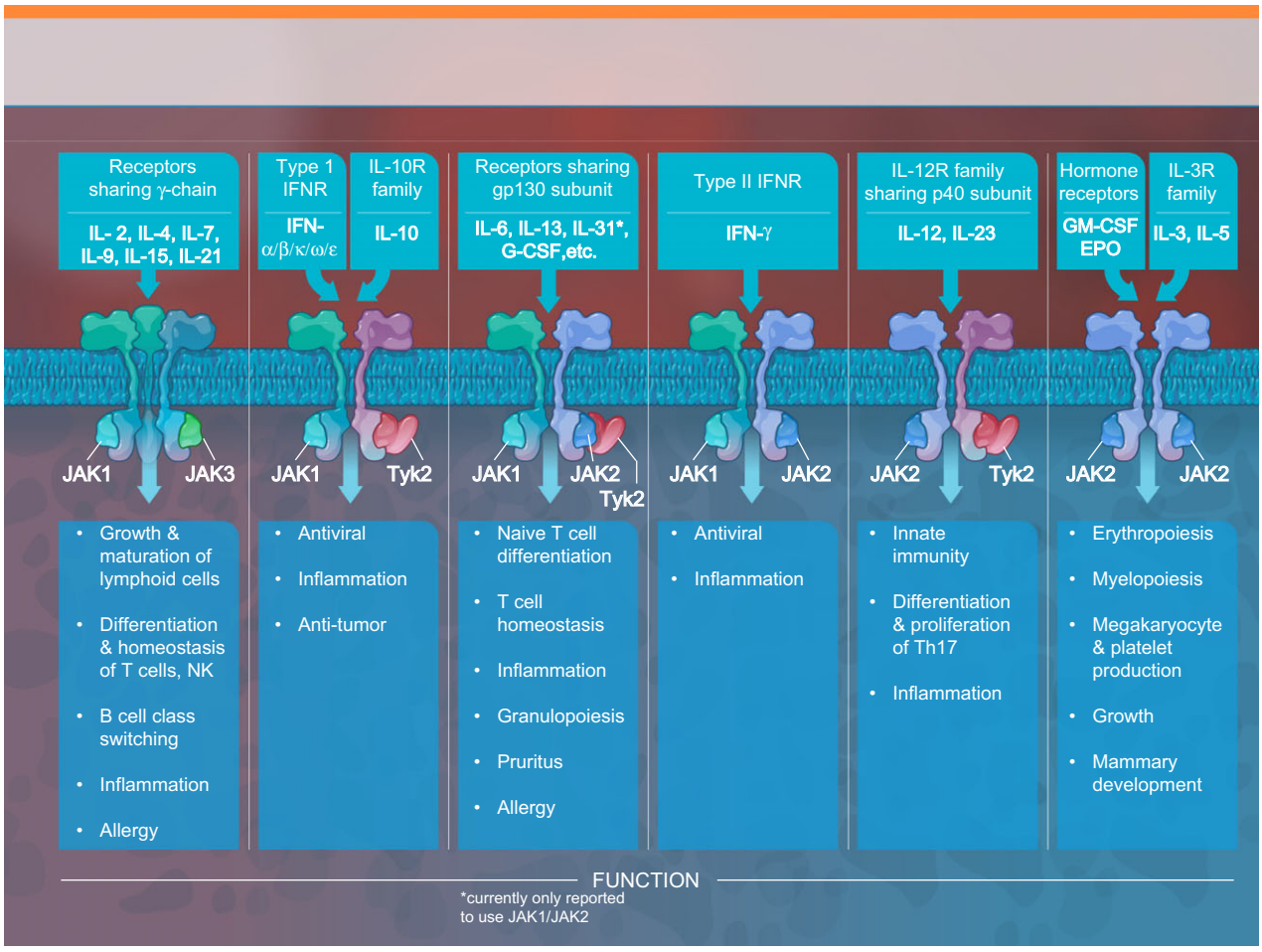

Fig. 2. Cytokine receptor families that utilize Janus kinase (JAK) enzymes for signaling. Cytokines are small proteins secreted by cells that can produce a variety of responses such as growth, development, differentiation, and activation of immune cells as well as nonimmune cell types. Cytokines exert these biological effects by binding to receptors on the surface of cells. Several cytokine receptors rely on the association and activation of JAK enzymes on the cytoplasmic portion of the receptor in order to transmit signals to the nucleus and induce necessary changes within the cell. Cytokine receptors can be groups according to the types of JAKs that are recruited to the receptor complexes. Many cytokines involved in allergy, inflammation, and pruritus bind receptor complexes that utilize JAK1. For example, IL-2 and IL-4 will bind receptor complexes that recruit JAK1 and JAK3. IL-6 and IL-13 bind receptors that engage JAK1, JAK2, and TYK2, and IL-31 will engage receptors that activate JAK1 and JAK2. In contrast, several cytokines involved in hematopoiesis (GM-CSF, erythropoietin) or innate immune cell defenses (IL-12 and IL-23) activate receptors dependent on JAK2/JAK2 or JAK2/TYK2 pairings.

nib potency and selectivity in canine cell model systems to determine whether this initially observed selectivity pattern toward JAK1-dependent cytokines will hold true in canine receptor systems, particularly those dependent on JAK2/TYK2 pairings.

Oclacitinib (APOQUEL®) was recently approved for the control/treatment of pruritus associated with allergic dermatitis and the control or treatment of AD in dogs at least 12 months of age in the US and EU. In the present study, oclacitinib was shown to inhibit JAK1-dependent cytokines involved in allergy, inflammation, and pruritus, suggesting that these are the mechanisms by which oclacitinib is effectively treating allergic skin diseases in dogs. We recently disclosed the results of a placebo-controlled field trial evaluating the efficacy of oclacitinib in client-owned dogs with allergic dermatitis (Cosgrove *et al.*, 2013). Oclacitinib produced a significant reduction in mean Owner Pruritus Visual Analog Scale (VAS) scores in these dogs compared with placebo-treated animals as early as Day 1 and on all other evaluation days of the study (Days 1–7; $P < 0.0001$). The Veterinary Dermatitis VAS scores were also significantly reduced on Day 7 ($P < 0.0001$) in the oclacitinib-treated animals compared with placebo. Therefore, inhibition of JAK1-dependent cytokines involved in allergy, inflammation, and pruritus is an effective and novel way to treat clinical signs associated with canine allergic skin disease.

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CONFLICT OF INTEREST

Zoetis employees are Zoetis Inc. shareholders.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Sequence homology between human and canine JAK family members.

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