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Research article

Gut-Selective Design of Orally Administered Izencitinib (TD-1473) Limits Systemic Exposure and Effects of Janus Kinase Inhibition in Nonclinical Species

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ABSTRACT

Izencitinib (TD-1473), an oral, gut-selective pan-Janus kinase (JAK) inhibitor under investigation for treatment of inflammatory bowel diseases, was designed for optimal efficacy in the gastrointestinal tract while minimizing systemic exposures and JAK-related safety findings. The nonclinical safety of izencitinib was evaluated in rat and dog repeat-dose and rat and rabbit reproductive and developmental toxicity studies. Systemic exposures were compared with JAK inhibitory potency to determine effects at or above pharmacologic plasma concentrations (≥1× plasma average plasma concentration [C_{ave}]:JAK 50% inhibitory concentration [IC_{50}] ratio). In rats and dogs, 1000 and 30 mg/kg/day izencitinib, respectively, produced minimal systemic findings (ie, red/white cell changes) and low systemic concentrations (approximately 1× plasma C_{ave}:JAK IC_{50} ratio) with an 8× nonclinical:clinical systemic area under the curve (AUC) margin compared with exposures at the highest clinically tested dose (300 mg, quaque die, once daily, phase 1 study in healthy volunteers). In dogs, it was possible to attain sufficient systemic exposures to result in immunosuppression characteristic of systemic JAK inhibition, but at high AUC margins (43×) compared with systemic exposures observed at the highest tested dose in humans. No adverse findings were observed in the gastrointestinal tract or systemic tissues. Izencitinib did not affect male or female fertility. Izencitinib did not affect embryonic development in rats and rabbits as commonly reported with systemic JAK inhibition, consistent with low maternal systemic concentrations (2–6× plasma C_{ave}:JAK IC_{50} ratio, 10–33× nonclinical:clinical AUC margin) and negligible fetal exposures. In conclusion, the izencitinib gut-selective approach resulted in minimal systemic findings in nonclinical species at pharmacologic, clinically relevant systemic exposures, highlighting the impact of organ-selectivity in reducing systemic safety findings.

Key words: JAK inhibitor; gut-selective; nonclinical safety.

Janus kinases (JAKs) encompass a family of nonreceptor tyrosine kinases (JAK1, JAK2, JAK3, and TYK2) intracellularly associated with types I and II cytokine receptors that act through signal transducer and activator of transcription (STAT) proteins to regulate gene transcription (Babon *et al.*, 2014; Ghoreschi *et al.*, 2009; Murray, 2007). Pharmacologic inhibition of JAK isoforms has been shown to be beneficial in multiple inflammatory and proliferative diseases such as myelofibrosis, rheumatoid arthritis, and inflammatory bowel diseases (Boland and Vermeire, 2017; Choy, 2019; Sandborn *et al.*, 2017; Seavey and Dobrzanski, 2012). Ruxolitinib was the first marketed JAK inhibitor in the United States (2011), followed by tofacitinib (2012), baricitinib (2018), and upadacitinib (2019). Though JAK inhibition has shown to be effective in these disease states, extensive changes in red and white cell parameters can limit the extent of therapeutic benefit achievable without corresponding adverse clinical effects.

Systemic JAK inhibition is accompanied by several dose-limiting toxicities (Figure 1) that can require clinical monitoring (Agrawal *et al.*, 2020). Hematopoietic abnormalities are of significant concern and have been observed both in nonclinical programs and in humans. Such toxicities include thrombocytopenia, neutropenia, lymphopenia, and anemia (Agrawal *et al.*, 2020; Choy 2019; European Medicines Agency, 2012, 2013, 2017a,b, 2019; U.S. Food and Drug Administration, 2011, 2012, 2018, 2019; Salas *et al.*, 2020). These JAK-associated immunosuppressive effects can progress to serious and opportunistic infections such as herpes zoster or reemergent tuberculosis (Agrawal *et al.*, 2020; Choy, 2019; Colombel, 2018; Salas *et al.*, 2020). To prevent such adverse events, health agencies have mandated black box warnings on the label as well as additional monitoring requirements (Agrawal *et al.*, 2020; Choy, 2019; Colombel, 2018; Gadina *et al.*, 2019; Salas *et al.*, 2020). Similarly, reduced immunosurveillance due to JAK inhibition has also raised concerns for secondary effects such as malignancy. Nonclinical studies have generally failed to inform malignancy risk, but tumorigenicity has been observed in some cases (European Medicines Agency, 2013, 2017a, 2019; Shuey *et al.*, 2016). Clinically, increased hematopoietic malignancies and specific types of solid tumors have been reported in patients receiving systemic JAK inhibitors (Agrawal *et al.*, 2020; Gadina *et al.*, 2019); however, a clear association between JAK inhibition and increased malignancy risk has not always manifested in large meta-analyses (Curtis *et al.*, 2016; Maneiro *et al.*, 2017). Early reports of a postmarketing safety study to assess major adverse cardiovascular events (MACEs) and malignancies with tofacitinib treatment failed to demonstrate noninferiority of tofacitinib versus tumor necrosis factor-alpha inhibitors (Pfizer, 2021). Most recently, the Food and Drug Administration (FDA) updated warnings of serious heart-related events, cancer, blood clots, and death with systemic JAK inhibition for certain chronic inflammatory conditions.

Other commonly reported safety findings with JAK inhibitor treatment include elevations in liver transaminases, creatinine and creatine phosphokinase, and hyperlipidemia as well as cases of gastrointestinal (GI) perforation, though no class-related warnings have been issued (Agrawal *et al.*, 2020; Bechman *et al.*, 2019; Choy, 2019; Salas *et al.*, 2020). Another emerging safety risk for this class of molecules has resulted from reports of rare but life-threatening thromboembolic events, including deep vein thrombosis and pulmonary embolism (Agrawal *et al.*, 2020; Mogul *et al.*, 2019; Sandborn *et al.*, 2019). The mechanisms leading to these serious adverse events are likely multi-factorial, such as potential for disruption of antithrombotic pathways via inhibition of cytokines associated with the JAK pathway, and currently are poorly defined with nonclinical studies conducted to date failing to elucidate the risk for such thromboembolic events. Notably, epidemiological data have suggested that patients with prior history of cardiovascular events and/or susceptibility factors may be at increased risk for thromboembolic events (Agrawal *et al.*, 2020; Gadina *et al.*, 2019; Vallejo-Yague *et al.*, 2020).

With respect to reproductive and developmental risks, nonclinical data have identified teratogenicity and decreased female fertility across the JAK inhibitor class, leading to recommended avoidance of JAK inhibitor use in pregnancy and during breastfeeding (Agrawal *et al.*, 2020; European Medicines Agency, 2012, 2013, 2017a b, 2019, 2020; U.S. Food and Drug Administration, 2011, 2012, 2018, 2019; Salas *et al.*, 2020). Effects on the male reproductive system and fertility have also been identified in nonclinical studies with the systemically available JAK inhibitor filgotinib, leading to concern for clinical effects on sperm parameters (European Medicines Agency, 2020; Gilead Sciences, Inc., 2020).

Izencitinib (TD-1473) is a small molecule pan-JAK inhibitor that was designed for gut selectivity, resulting in high local GI tissue exposure and minimal systemic exposure following oral administration. This profile was designed to maximize the pharmacologic effect in the GI tract and mitigate systemic JAK effects based on the hypothesis that low systemic exposures would minimize the risk of immunosuppression, malignancy, and perhaps thrombotic effects due to reduced systemic JAK inhibition. Nonclinical gut-selective exposure and biological activity was previously demonstrated in rodent studies that exhibited high colonic tissue concentrations, low systemic exposure, and biological activity in an *in vivo* colitis model (Sandborn *et al.*, 2020). In a phase 1b clinical trial in ulcerative colitis patients, izencitinib has also demonstrated a favorable clinical safety profile with low systemic exposures, high intestinal exposure, and exhibited clinical response assessed by total Mayo score and endoscopic improvement (Sandborn *et al.*, 2020).

The systemic safety risks associated with JAK inhibitors can be dose-limiting and thus an impediment to fully exploiting the antiinflammatory effects of JAK inhibition in the tissue of interest. As such, the studies reported herein describe the nonclinical safety assessment of izencitinib including general toxicity studies in 2 pharmacologically relevant species, rat and dog, and reproductive and developmental toxicity studies in rats and rabbits. These studies sought to probe the safety profile resulting from the gut-selective design approach (Figure 1) at nonclinical doses producing both pharmacologically active (approximately 1× plasma average plasma concentration [C_{ave}]:JAK 50% inhibitory concentration [IC_{50}] ratio) and suprapharmacologic systemic concentrations.

MATERIALS AND METHODS

Test Article

Izencitinib (TD-1473, ≥98.4% HPLC purity) was synthesized by Theravance Biopharma US, Inc. (South San Francisco, California, USA) and formulated for oral administration in a vehicle consisting of 1% (w/v) Methocel E5 Premium (low viscosity) and 0.1% (v/v) Tween-80 in ultrapure water.

Animal Care and Use

All studies were conducted in AALAC-accredited facilities and performed in accordance with the National Research Council

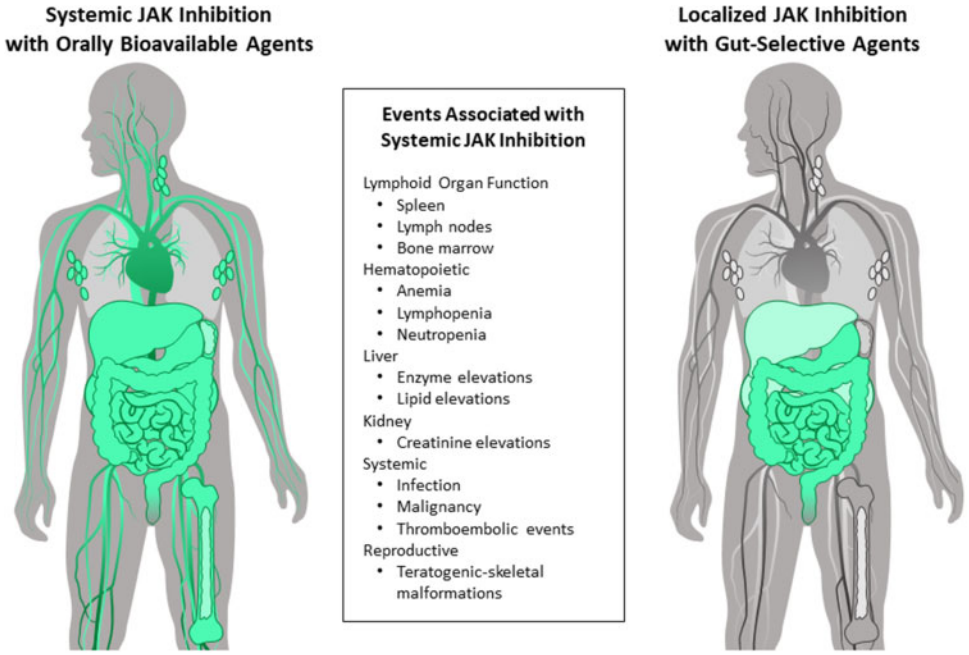

Figure 1. Potential mitigation of events associated with systemic Janus kinase (JAK) inhibition using a gut-selective approach. Distribution of orally bioavailable versus gut-selective agents is shown in green. Effects associated with systemic JAK inhibition are listed and included as mitigation opportunities for a gut-selective approach.

Guide for the Care and Use of Laboratory Animals and in compliance with Good Laboratory Practice (GLP) regulations. All study protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the testing facilities. Male and/or female Crl:CD Sprague Dawley (SD) rats were obtained from Charles River Laboratories (either Quebec, Canada or Raleigh, North Carolina), for use in the repeat-dose toxicity studies, the embryo–fetal development (EFD) toxicity study, and the fertility and early embryonic development (FEED) toxicity study. Male and female beagle dogs were obtained from Marshall BioResources (North Rose, New York) or Covance Research Products, Inc. (Cumberland, Virginia) for use in the repeat-dose toxicity studies. Female Hra:(NZW) SPF New Zealand White rabbits were obtained from Covance Research Products, Inc. (Greenfield, Indiana) for the EFD toxicity study.

Repeat-dose General Toxicity Study Design

Dose formulations (0 [vehicle], 30, 100, or 1000 mg/kg/day izencitinib) were administered daily to all rats (7–8 weeks old at study start) via oral gavage (10 ml/kg) for 4, 13, or 26 weeks. All studies included a main dosing phase ($n = 10$/sex/dose group), a 4-week dose-free recovery phase to determine potential reversibility of any findings ($n = 5$/sex in vehicle and high izencitinib dose groups in all studies plus $n = 5$/sex in low and mid izencitinib dose groups in the 13-week study), and a toxicokinetic phase ($n = 3$/sex vehicle dose group; $n = 7$/sex low, mid, and high izencitinib dose groups). Study endpoints included clinical observations, body weight, food consumption, ocular examinations, clinical pathology, organ weights, and histopathology of standard tissues.

Dose formulations of izencitinib were administered daily to dogs via oral gavage (10 ml/kg) for 4, 13, or 39 weeks. In the 4-week study, dogs received 0 (vehicle), 10, 30, or 300 mg/kg/day izencitinib. Due to adverse clinical observations in the 4-week study, the highest dose of 300 mg/kg/day izencitinib was reduced to 100 mg/kg/day izencitinib beginning on days 13 and 14 in females and males, respectively. Dogs received 0 (vehicle), 10, 30, or 100 mg/kg/day izencitinib for the duration of the subsequent 13- and 39-week studies. Dogs were 10–11 months old at the start of the 4- and 13-week studies, and 7–8 months at the start of the 39-week study. All studies included a main dosing phase ($n = 3$–4/sex/dose group), which incorporated toxicokinetics, and a 4-week dose-free recovery phase ($n = 2$/sex/dose group). Study endpoints included clinical observations, body weight, food consumption, ocular examinations, electrocardiography assessments, clinical pathology, organ weights, and histopathology of standard tissues.

Rats and dogs in all repeat-dose general toxicity studies were fasted overnight before blood sampling, and food and water were withheld during urine collection. Blood samples for analysis of hematology, coagulation, and clinical chemistry parameters were collected from all animals prior to study start to establish baseline parameters, at the end of the main dosing phase, and at the end of the recovery phase in each study. For the 39-week dog study, interim samples were also collected from all dose groups at weeks 28 and 32 for the control and high izencitinib dose group. Urinalysis samples were collected from all rats and dogs at the end of the main dosing phase and at the end of the recovery phase in each study. Peripheral blood immunophenotyping was performed via a validated flow cytometry method at the end of the main dosing phase and at the end of the recovery phase in the 39-week dog study.

FEED Toxicity Study Design

Dose formulations (0 [vehicle], 30, 100, or 1000 mg/kg/day izencitinib) were administered daily via oral gavage (10 ml/kg) to male and female rats ($n = 22$/sex/dose group) beginning 15 days prior to cohabitation and throughout a 14-day cohabitation period, with izencitinib administration to females continuing up to gestation day (GD) 7. Any females that had not mated at the completion of the cohabitation period were designated as being at GD 0 and continued to receive their respective dose formulation

for an additional 8 days. Clinical observation, food consumption, and body weight data were collected throughout the study. Estrous cycling in females was monitored by vaginal cytology beginning 14 days prior to cohabitation and continued until spermatozoa were observed in the vaginal cytology samples or a copulatory plug was observed. Gross necropsy of male and female reproductive tracts was performed with histopathological analysis. Reproductive parameters were determined as described in (Denny and Faqi, 2017).

EFD Toxicity Study Design

Time-mated female rats (10–12 weeks old at start of dosing) were administered dose formulations of 0 (vehicle), 30, 100, or 1000 mg/kg/day izencitinib via daily, oral gavage (10 ml/kg) during the period of organogenesis, GDs 6 through 17. Time-mated female rabbits (6–8 months old at start of dosing) were administered dose formulations of 0 (vehicle), 10, 30, or 60 mg/kg/day izencitinib via daily oral gavage (10 ml/kg) during the period of organogenesis, GDs 7 through 19. Each dose group in both the rat and rabbit studies consisted of a main toxicity phase ($n = 22$/dose group) and a toxicokinetic phase ($n = 3$, vehicle group; $n = 9$ [rat] or 6 [rabbits]/dose group for low, mid, and high izencitinib doses). Clinical observation, food consumption, and body weight data were collected throughout the studies. Cesarean sections and gross necropsies were performed on GD 21 (rats) or GD 29 (rabbits). Uteri were excised, weighed, and examined for number and placement of viable and nonviable fetuses, early or late resorptions, and any abnormalities, and ovaries were examined for number of corpora lutea. Fetal analyses include body weights and examination for external and visceral abnormalities. Skeletal evaluations, including bone alignment and degree of ossification assessments, were performed following evisceration and Alizarin Red S staining.

Bioanalysis and Toxicokinetics

For all repeat-dose toxicity studies, approximately 0.2 ml (rats) or 0.5–1.0 ml (dogs) blood was collected via jugular venipuncture at 0, 0.5, 1, 3, 6, 9, and 24 h postdose on day 1 and at the end of the main dosing phase. Interim analysis was also conducted on day 197 in the 39-week dog study. For the EFD studies, approximately 0.2 ml blood was collected via jugular venipuncture from maternal rats at 0, 0.5, 1, 3, 6, 9, and 24 h postdose on GDs 6 and 17, and 0.5 ml blood was collected via medial auricular arterial puncture from maternal rabbits at 0, 0.5, 1, 3, 6, 9, and 24 h postdose on GDs 7 and 19. Approximately 0.2 ml (rats) or 0.5 ml (rabbits) fetal blood (collected from more than one randomly selected fetus from 3 litters/dose group to achieve total target volume) was collected via cardiac puncture at 3, 9, and 24 h postdose or 3 and 24 h postdose in rats and rabbits, respectively, at the end of the dosing period (including 3 h for control group).

All plasma samples were analyzed for izencitinib concentration via liquid chromatography tandem mass spectrometry using a validated analytical procedure and data collected using Analyst from AB Sciex. Toxicokinetic parameters (maximal plasma exposure [C_{max}] and area under the curve [$AUC_{0–24}$]) were estimated via Phoenix WinNonlin software (Pharsight Corporation, Mountain View, California) using a noncompartmental approach consistent with the oral route of administration. C_{ave} values were determined in Microsoft Excel based on $AUC_{0–24}$ data. C_{ave} values were then compared with human cellular JAK IC_{50} ranges (Sandborn *et al.*, 2020):uncorrected (12.73–80.31 ng/ml) and human protein binding corrected (87.18–550.1 ng/ml) using a human plasma protein binding correction of 85.4% (data not shown). Composite izencitinib toxicokinetic profiles were reported for rats and rabbits with a minimum $n = 3$/sex/dose/time point in all repeat-dose toxicity studies and EFD studies, whereas serial izencitinib toxicokinetic profiles were reported for dogs in all repeat-dose toxicity studies.

Histopathology

All preserved tissues across all studies were embedded in paraffin, sectioned, and slides prepared followed by staining in hematoxylin and eosin. Microscopic evaluation of all slides was performed by a board-certified veterinary pathologist with independent peer review.

Statistical Analyses

Descriptive statistics (mean and SD) were calculated for all numerical data with respect to sex. Analysis of izencitinib toxicokinetic data determined a lack of sex differences in izencitinib exposures in rat and dog repeat-dose toxicity studies. As such, male and female data were combined, and descriptive statistics calculated for each sex-averaged dose group. Homogeneity of group variances was evaluated using either Shapiro–Wilk or Levene's test. All dose groups administered izencitinib were compared with the vehicle-treated group in each study. All data that met parametric assumptions were compared via 1-way analysis of variance (ANOVA) with Dunnett's pairwise analysis or unpaired *t* test for analyses. Nonparametric data were compared via Kruskal–Wallis test with Dunn's pairwise analysis or Mann–Whitney test. Proportional data, such as that acquired in the FEED and EFD toxicity studies, were analyzed via Fisher's exact test. Fetal weights in the EFD toxicity studies were analyzed via analysis of covariance with Dunnett's pairwise analysis and litter size as the covariate. All data were reported at the 0.1, 1, and 5% significance level.

RESULTS

Izencitinib Tolerability with Repeated Administration in Rats and Dogs

Exploratory non-GLP compliant studies in both rats and dogs were conducted to select doses for subsequent pivotal studies with the highest dose in each species based on tolerability after 7 days of repeated izencitinib administration.

In rats, izencitinib was well-tolerated up to the highest tested dose of 1000 mg/kg/day with minimal findings in pivotal 4-, 13-, and 26-week studies. There were no effects on food consumption, ophthalmic evaluations, clinical chemistry, or urinalysis parameters in rats.

In the 4-week dog study, izencitinib was evaluated up to 300 mg/kg/day; however, the 300 mg/kg/day dose level was not tolerated after 12–13 days based on adverse clinical observations (ie, emesis, decreased activity, or coordination, hyperthermia, and/or weakness), leading to dose reduction to 100 mg/kg/day and early euthanasia of 3 dogs on days 18 and 19. In the 13-week dog study, izencitinib was evaluated up to 100 mg/kg/day with clinical observations limited to changes in fecal consistency across all dose levels and occasional emesis. Izencitinib was also evaluated up to 100 mg/kg/day in the 39-week study; however, the 100 mg/kg/day dose level was not tolerated and resulted in early euthanasia of 2 dogs at 25–26 weeks due to clinical observations in the skin. Particularly, macroscopic findings of masses, discolorations, and abrasions correlated microscopically with

papillomas associated with canine papillomavirus infection or dermal inflammation associated with infestation by *Demodex* spp. These dermal findings were considered secondary to izencitinib pharmacologic immunomodulatory effects. The main dosing phase for the 100 mg/kg/day dose level was subsequently terminated at 29 weeks ($n = 6$ remaining dogs) with immediate initiation of the recovery phase for that dose level ($n = 4$ dogs). At the end of the recovery period, the dermal inflammation associated with *Demodex* spp. at 100 mg/kg/day was still noted, but no papillomas were present. As a result of early termination, data shown for the 100 mg/kg/day dose level in the 39-week study is at week 28 (date of data collection most proximal to dose level termination); data from all other dose levels shown are at week 39. Other clinical observations at the 100 mg/kg/day dose level in the 39-week dog study included non-adverse alterations in fecal consistency with no histological correlate. No effects on ophthalmology or electrocardiography endpoints, food consumption, or urinalysis parameters were observed across all dog studies.

Izencitinib Toxicokinetics Following Daily Administration in Rats and Dogs

Mean plasma toxicokinetic parameters (C_{max}, $AUC_{0–24}$, and C_{ave}) on the last day of dosing for each rat and dog repeat-dose toxicity study are shown in Table 1. Systemic concentrations are plotted in Figure 2 against the izencitinib human cellular JAK IC_{50} ranges (uncorrected [12.73–80.31 ng/ml] and human plasma protein binding corrected [87.18–550.1 ng/m;] JAK1, JAK2, JAK3, and TYK2 IC_{50}) to provide pharmacologic relevance of the observed exposures (Sandborn *et al.*, 2020). Previous nonclinical and clinical studies established that the tofacitinib plasma C_{ave} and the time tofacitinib plasma concentrations spent above the JAK IC_{50} threshold as the primary determinant of efficacy, with 8–12 h above JAK whole blood IC_{50} being optimal regardless of BID or QD) dosing (Dowty *et al.*, 2014). A similar time over JAK IC_{50} threshold for izencitinib was determined for each dose level in the repeat-dose toxicity studies using available human cellular IC_{50} values to provide a benchmark against any observed systemic effects. JAK IC_{50} values in nonclinical species were unavailable but expected to be similar based on species homology. Uncorrected human JAK IC_{50} values were selected as the minimum threshold to designate exposures as supratherapeutic as this provided a more conservative estimate, with a comparison to human plasma protein-corrected JAK IC_{50} values also provided for reference.

Systemic izencitinib exposures (mean $AUC_{0–24}$) as well as the maximum and average systemic concentrations (C_{max} and C_{ave}, respectively) generally increased with dose from 30 to 1000 mg/kg/day in both male and female rats across all studies. There were no clear differences between male and female rats in any of the toxicokinetic parameters assessed, with mean values generally within 2-fold. Peak concentrations were between 0.5 and 3 h across all doses in each rat study, regardless of sex. Plasma izencitinib concentrations in rats were plotted against total and human plasma protein binding corrected (gray-and green-shaded areas, respectively; Figure 2A) human JAK IC_{50} values (Sandborn *et al.*, 2020). Plasma izencitinib exposures at the 1000 mg/kg/day dose level in rats achieved supratherapeutic levels for 9–24 h with a plasma C_{ave}:uncorrected JAK IC_{50} ratio of 1–3× (plasma C_{ave}:protein-corrected JAK IC_{50} ratio <1). Plasma concentrations at the 100 mg/kg/day dose level only briefly rose above the uncorrected human JAK IC_{50} threshold, while the 30 mg/kg/day izencitinib dose resulted in subtherapeutic plasma concentrations (<1× plasma C_{ave}:uncorrected and protein-corrected JAK IC_{50} ratio).

Table 1. Summary of Rat and Dog Total Izencitinib Plasma Toxicokinetic Parameters

Species	Dosing duration (weeks)	Dose (mg/kg/day)	Mean C_{max} (ng/ml)[a]		Mean $AUC_{0–24}$ (ng·h/ml)[a]		Mean C_{ave} (ng/ml)[a]	
			Male	Female	Male	Female	Male	Female
Rat[b]	4	30	2.88	4.82	23.4	25.7	0.975	1.07
		100	9.96	13.1	71.3	70.5	2.97	2.94
		1000[c]	62.5	91.8	335	384	14.0	16.0
	13	30	6.53	3.54	58.2	54.6	2.43	2.28
		100	8.55	37.4	131	227	5.46	9.46
		1000[c]	71.9	278	452	1530	18.8	63.8
	26	30	4.52	6.02	34.6	16.7	1.44	0.700
		100	15.8	32.5	95.7	127	3.99	5.29
		1000[c]	60.6	96.3	384	453	16.0	18.9
Dog[d]	4	10	8.11	10.4	55.2	45.5	2.30	1.90
		30[c]	93.4	64.5	459	356	19.1	14.8
		300/100[e]	247	314	1470	2680	61.3	112
	13	10	6.52	7.37	38.6	36.7	1.61	1.53
		30[c]	82.7	46.9	300	260	12.5	10.8
		100	522	368	4550	2320	190	96.7
	39	10	7.50	11.5	49.9	74.6	2.08	3.11
		30[c]	70.3	81.7	382	491	15.9	20.5
		100[f]	304	545	1670	3140	69.6	131

Mean total plasma toxicokinetic parameters on the final day of dosing are shown in rats and dogs following 4, 13, 26 (rats only), or 39 (dogs only) weeks of daily, oral izencitinib administration.

[a]Steady-state maximal plasma exposure (C_{max}), area under the curve $(AUC)_{0–24}$, and average plasma concentration (C_{ave}) on the last day of dosing.

[b]Data are derived from mean composite toxicokinetic profiles for each dose.

[c] The no observed adverse effect level determined in each study.

[d]Data are derived from individual toxicokinetic profiles for each dose.

[e]The 300 mg/kg/day dose level was not tolerated after 12–13 days, dosing was reduced to 100 mg/kg/day.

[f]Week 28 (day 197) steady-state C_{max}, $AUC_{0–24}$, and C_{ave} shown due to termination of 100 mg/kg/day dose group at week 29.

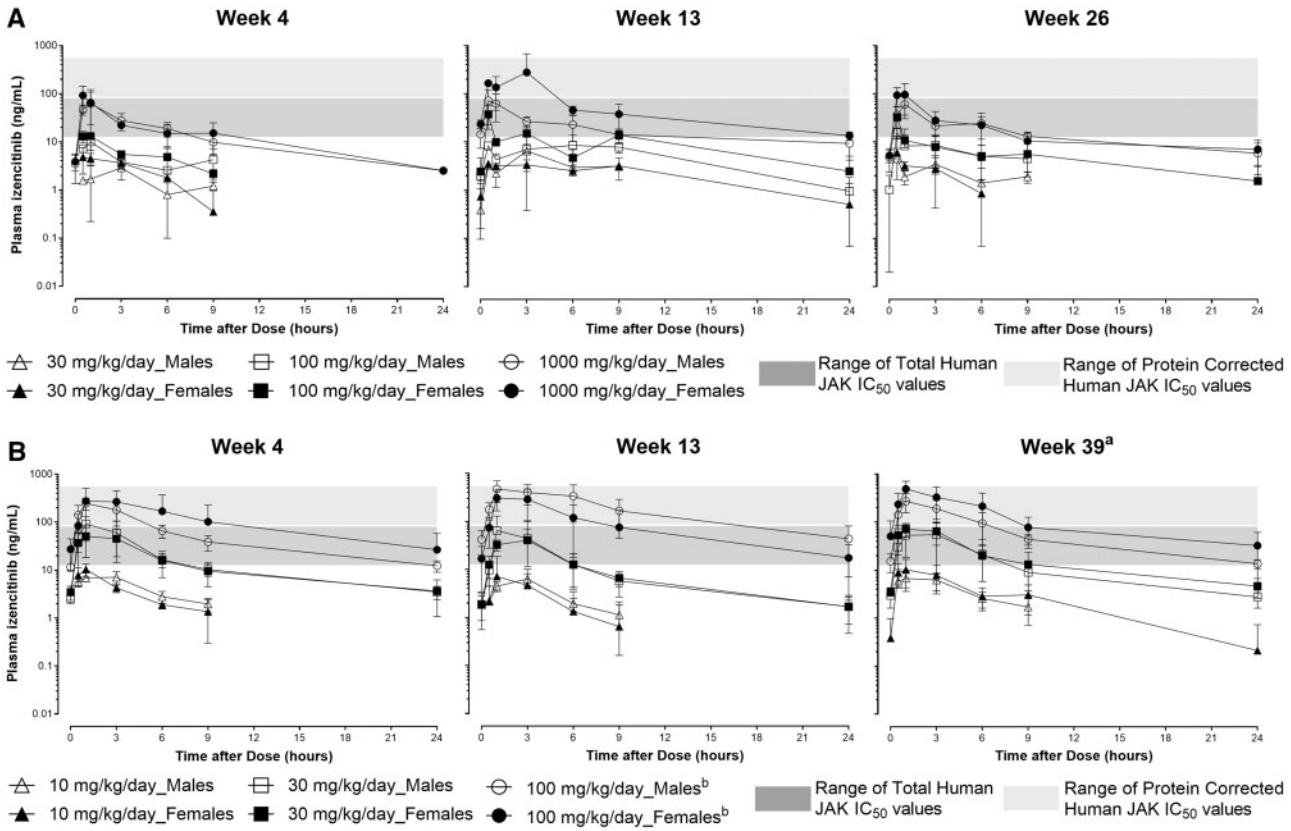

Figure 2. Steady-state plasma exposure profiles following daily izencitinib administration in rats and dogs. Izencitinib plasma exposure profiles on final day of dosing in rats (A) and dogs (B) following 4, 13, 26 (rats only), or 39 (dogs only) weeks of daily, oral izencitinib administration plotted against uncorrected (12.73–80.31 ng/ml) and human plasma protein binding corrected (87.18–550.1 ng/ml) human cellular Janus kinase 50% inhibitory concentration ranges (Sandborn *et al.*, 2020). [a]Day 197 (week 28) izencitinib plasma concentrations shown for 100 mg/kg/day dose due to early termination of the dose group at week 29. [b]In the 4-week study dogs were given 300 mg/kg/day from days 1 to 12 (females) or days 1–13 (males). Due to adverse clinical observations, the high dose of 300 mg/kg/day was lowered to 100 mg/kg/day.

Similar to rats, systemic izencitinib exposures (mean AUC_{0-24}) and maximum and average systemic concentrations (C_{max} and C_{ave}, respectively) in dogs generally increased with dose across all studies. There were no clear differences between sexes or evidence of accumulation with all parameters falling within 2-fold. Peak plasma concentrations in male and female dogs occurred between 1 and 3 h across all doses in each study. Plasma izencitinib concentrations in dogs were also plotted against uncorrected and human plasma protein binding corrected (gray- and green-shaded areas, respectively; Figure 2B) human JAK IC_{50} values (Sandborn *et al.*, 2020). At the highest tested dose in dogs, 100 mg/kg/day, plasma izencitinib concentrations were supratherapeutic for up to 24 h across all studies with a plasma C_{ave}:uncorrected JAK IC_{50} ratio of 7–11× (1–2× plasma C_{ave}:protein-corrected JAK IC_{50} ratio). At the mid-dose, 30 mg/kg/day, plasma izencitinib levels were within the therapeutic range (1× plasma C_{ave}:uncorrect JAK IC_{50} ratio; <1× plasma C_{ave}:protein-corrected JAK IC_{50} ratio) for approximately 6–9 h, whereas the lowest dose, 10 mg/kg/day izencitinib, resulted in subtherapeutic plasma izencitinib concentrations (<1× plasma C_{ave}:uncorrected and protein-corrected JAK IC_{50} ratio).

Izencitinib Effects in Rats and Dogs were Limited to Pharmacology of JAK Inhibition

No sex-related differences in exposure to izencitinib were identified in either rats or dogs across all repeat-dose toxicity studies. Additionally, no clear sex-specific effects on clinical pathology parameters or organ weights were identified. Due to a lack of discernible dose-related, sex-specific effects, male and female clinical pathology, organ weight, and immunophenotyping data were averaged for simplification of presentation and analyzed for significant izencitinib effects across dose groups. Clinical pathology parameters of note due to consistently observed changes, specifically white blood cell, lymphocyte, lymphocyte subsets (immunophenotyping, dogs only), neutrophil, platelet, red blood cell, hemoglobin, hematocrit, and reticulocyte levels, as well as organ weight and histology data are described below.

In rats, changes in hematology parameters observed in the repeat-dose toxicity studies were related to the pharmacology of izencitinib and considered nonadverse due to the low magnitude of change and limited microscopic correlates. Izencitinib effects on red and white blood cell parameters in rats during the main dosing phase in each study are shown in Figures 3A and 3B, respectively. Recovery phase data (reversibility) are described, but not plotted. No changes in red blood cell parameters were noted at any dose with 4 weeks of daily izencitinib administration to rats. A slight, reversible decrease in red blood cell counts (0.96×) was observed with 13 weeks of 1000 mg/kg/day izencitinib administration, whereas hemoglobin levels also were slightly decreased and remained so at the end of the recovery period (0.97× and 0.95×, respectively). In the 26-week study, hemoglobin levels were slightly decreased (0.95×) at all dose levels and remained reduced at the 1000 mg/kg/day dose level following the recovery period. A slight, reversible reduction (0.95×) in hematocrit also was observed in the 26-week

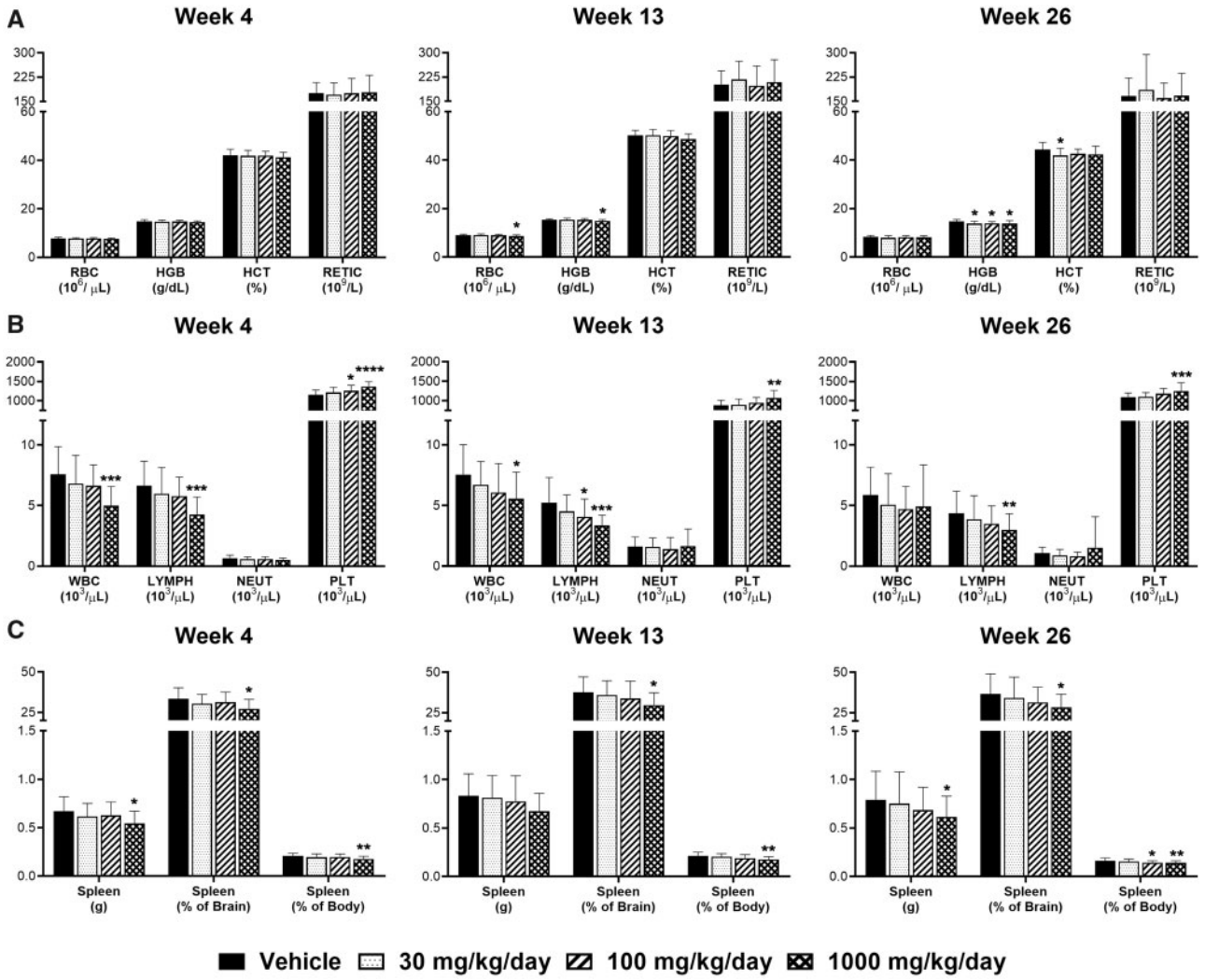

Figure 3. Izencitinib effects on hematology parameters and organ weight changes in rats. Red (A) and white (B) blood cell parameters and organ weight changes (C) following 4, 13, or 26 weeks of daily, oral izencitinib administration. Asterisks indicate significant difference from vehicle: $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, and $^{****}p < .0001$.

study at 30 mg/kg/day with no significant effects at other dose levels. No changes in reticulocyte levels were observed at any dose across the 3 rat studies. Reversible decreases in white blood cell (0.66×) and lymphocyte (0.64×) counts were observed with 4 weeks of 1000 mg/kg/day izencitinib administration in rats. Additionally, a slight reversible increase in platelet counts was observed at the 100 mg/kg/day (1.1×) and 1000 mg/kg/day (1.2×) izencitinib dose levels. Following 13 weeks daily izencitinib administration, a reversible decrease in white blood cell counts (0.74×) and a reversible increase in platelet counts (1.2×) was observed at the 1000 mg/kg/day izencitinib dose level. Reversible decreases in lymphocyte counts also were observed with 100 mg/kg/day (0.78×) and 1000 mg/kg/day (0.64×) izencitinib administration. Similar changes in white blood cell (0.68×) and platelet (1.1×) counts were observed in the 26-week rat study with 1000 mg/kg/day izencitinib administration. No izencitinib-related effects on neutrophils were observed at any dose in the 3 rat studies. Izencitinib-related effects on rat organ weights were limited to nonadverse, primarily reversible reductions in spleen weight at the 1000 mg/kg/day (0.78–0.86×) and 100 mg/kg/day (0.87×) dose levels (Figure 3C). No correlating microscopic changes were observed in either the 4- or 13-week studies. Reversible, minimally decreased lymphoid cellularity (4 of 10 males, 1 of 10 females) was observed at the 1000 mg/kg/day izencitinib dose level in the 26-week study, which correlated to the minor changes observed in circulating white and red blood cells described above. No other changes in organ weights or microscopic effects of izencitinib, including GI tissues, were observed up to the highest tested dose (1000 mg/kg/day) across all studies.

In dogs, izencitinib-related changes in white and red blood cell parameters (Figs.. 4A and 4B, respectively) were consistent with JAK inhibition. Changes in white and red blood cell parameters were observed at 300/100 (graphed as 100 mg/kg/day to reflect the dose reduction that occurred during the study), 100, and ≥30 mg/kg/day in the 4-, 13-, and 39-week studies, respectively. Recovery phase data are described, but not plotted. Reversible decreases in red blood cell parameters (0.82–0.85×) were observed at the 100 mg/kg/day dose level in the 4- and 13-week studies. No changes in reticulocyte levels were observed at any dose in the 4- and 13-week studies. In the 39-week study, red blood cell parameters were reduced at the 30 mg/kg/day (0.90–0.91×) and 100 mg/kg/day (0.76–0.79×) izencitinib dose levels. The observed reductions were largely reversible; however, hemoglobin levels (0.87×) and hematocrit (0.90–0.92×) remained reduced at the 100 mg/kg/day dose level and both dose levels,

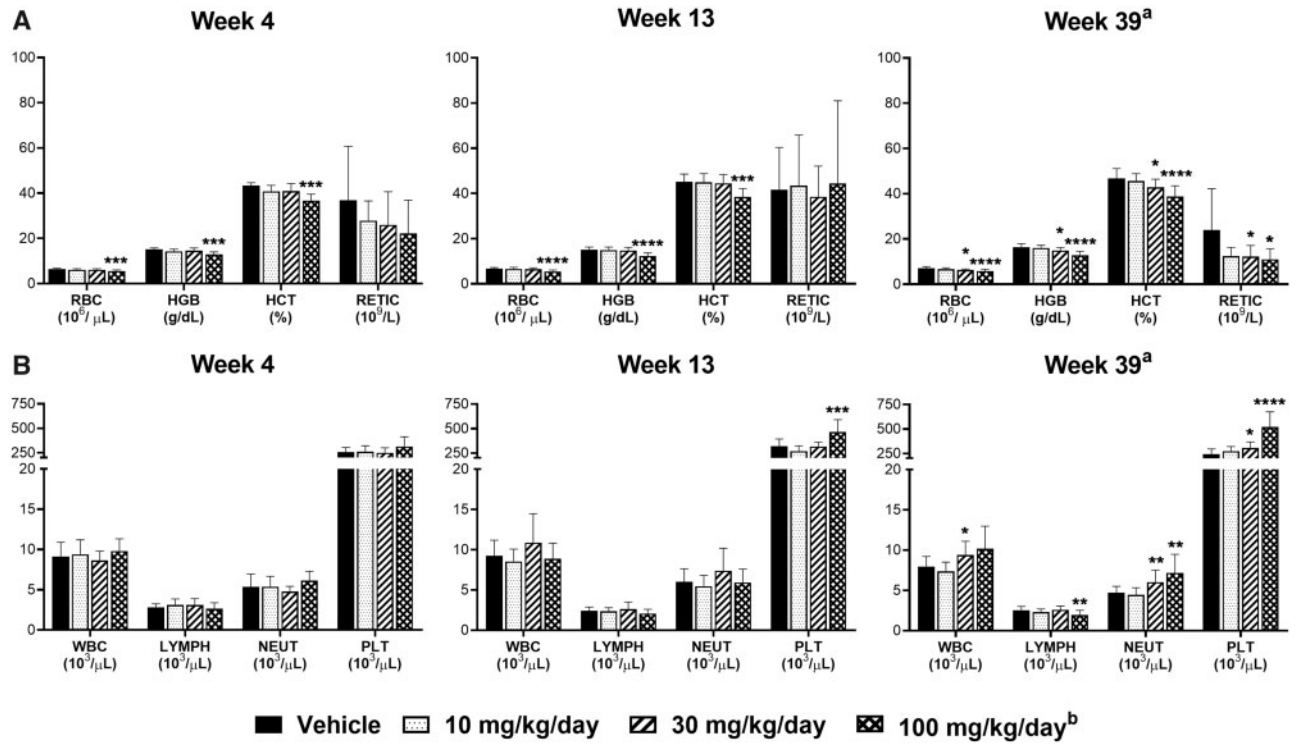

Figure 4. Izencitinib effects on hematology parameters in dogs. Red (A) and white (B) blood cell parameters following 4, 13, or 39 weeks of daily, oral izencitinib administration. Asterisks indicate significant difference from vehicle: *$p < .05$, **$p < .01$, ***$p < .001$, and ****$p < .0001$. [a]Week 28 values shown for 100 mg/kg/day dose due to termination of the dose group at week 29. [b]In the 4-week study dogs were given 300 mg/kg/day from days 1 to 12 (females) or days 1–13 (males). Due to adverse clinical observations, the high dose of 300 mg/kg/day was lowered to 100 mg/kg/day.

respectively. Reticulocytes were reversibly reduced (0.45–0.51×) with 30 mg/kg/day and 100 mg/kg/day izencitinib administration in the 39-week study. No significant effects on white blood cell parameters were observed at the end of the dosing phase in the 4-week dog study. No izencitinib-related effects on white blood cell, lymphocyte, or neutrophil counts were observed with 13 weeks izencitinib administration; however, a reversible increase (1.5×) in platelets was observed at the 100 mg/kg/day izencitinib dose level. In the 39-week study, white blood cell counts were mildly elevated (1.2×) at 30 mg/kg/day izencitinib at the end of the dosing phase. Though the effect was reversed in the recovery phase, a significant elevation (1.3×) was subsequently noted at the 100 mg/kg/day izencitinib dose level in recovery animals. A reversible decrease (0.70×) in lymphocyte counts also was observed at 100 mg/kg/day following 39 weeks izencitinib administration. Neutrophils (1.3–1.5×) and platelets (1.3–2.2×) were increased at 30 and 100 mg/kg/day izencitinib in the 39-week study with reversibility observed at the 30 mg/kg/day dose level. Immunophenotyping of specific lymphocyte subpopulations was performed in the 39-week dog study and presented in Figure 5. Effects on lymphocyte subsets were limited to the 100 mg/kg/day dose level. Specifically, reversible decreases in total lymphocytes (0.70×), total T cells (0.71×), and helper T cells (0.71×) were observed. Additionally, B cells were reduced (0.59×) without reversibility in the 4-week recovery phase. Changes in clinical chemistry parameters in dogs (data not shown) were inconsequential and limited to nontolerated doses (≥100 mg/kg/day) in the 4- and 13-week studies. All other clinical pathology changes were considered unrelated to izencitinib based on low and/or sporadic incidence, and/or similar incidence in controls. Microscopically, findings in dogs were consistent with JAK inhibition and included decreased hematopoietic and lymphoid cellularity in the bone marrow, gut-associated lymphoid tissue (GALT), mandibular and mesenteric lymph nodes, spleen, and/or thymus at ≥100 mg/kg/day in the 4- and 13-week studies and ≥30 mg/kg/day in the 39-week study. Additionally, reversible cortical adrenal vacuolation was observed at 100 mg/kg/day in the 39-week study and considered likely secondary to stress. The decreased cellularity in lymphohematopoietic tissues observed at the high-dose levels in dogs

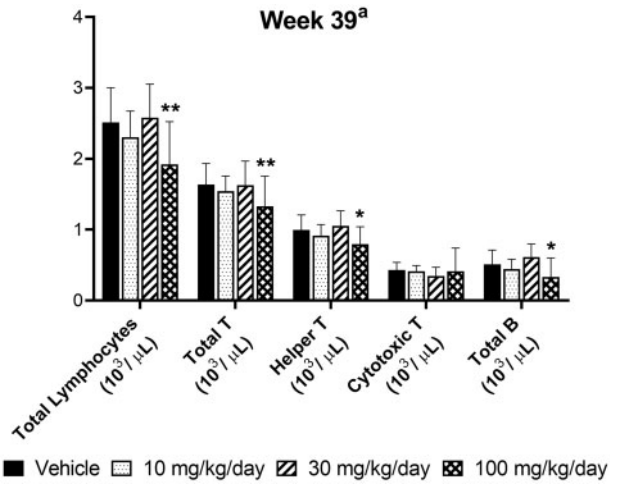

Figure 5. Lymphocyte phenotyping following 39-weeks izencitinib administration to dogs. Lymphocyte populations following 39 weeks of daily, oral izencitinib administration. Asterisks indicate significant difference from vehicle: *$p < .05$, and **$p < .01$. [a]Week 28 values shown for 100 mg/kg/day dose in dogs due to termination of the dose group at week 29; significance with respect to controls at week 28 are shown.

was consistent with izencitinib pharmacology, generally reversible, and thus was considered nonadverse. There were no adverse microscopic findings in GI tissues up to the highest tested dose (100 mg/kg/day) in all 3 dog studies.

No Izencitinib-Related Effects on Coagulation Parameters in Rats and Dogs

Sex-averaged coagulation data (prothrombin time, activated partial thromboplastin time, and fibrinogen) following daily izencitinib administration to rats and dogs are shown in Figures 6A and 6B, respectively. There were no changes in coagulation parameters at any dose in all 3 rat studies. No effects on prothrombin time or activated partial thromboplastin time were observed at any dose in the 3 dog studies. Fibrinogen levels were increased (1.3–1.4×) at the 100 mg/kg/day dose level in the 13- and 39-week dog studies. The elevations in fibrinogen in the absence of significant effects on prothrombin and activated partial thromboplastin times were considered related to the presence of dermal inflammation at this dose level, rather than a direct effect on coagulative capacity.

No Observed Adverse Effect Level Following Chronic Izencitinib Administration to Rats and Dogs

Based on the absence of adverse findings, the no observed adverse effect level (NOAEL) after 26 weeks of repeated izencitinib administration to rats was determined to be 1000 mg/kg/day izencitinib, corresponding to a 1× plasma C_{ave}:uncorrected JAK IC_{50} ratio. The dose-limiting finding in dogs was dermal inflammation in the 39-week study, and the NOAEL was thus considered 30 mg/kg/day izencitinib (1× plasma C_{ave}:uncorrected JAK IC_{50}).

No Izencitinib Effects on Fertility or EFD in Rats and Rabbits

The effects of izencitinib on gonadal function, mating behavior, and reproductive performance were determined in a FEED toxicity study in rats via daily oral gavage up to 1000 mg/kg/day. Mating performance parameters including number of females mated, pregnancy rate, mating index, and fertility index are summarized in Table 2. There were no changes in any of the study parameters evaluated. As a result of the lack of reproductive effects in the FEED study, the NOAEL was determined to be 1000 mg/kg/day.

The potential for izencitinib to produce maternal and/or embryo–fetal toxicity was determined in 2 EFD toxicity studies. Izencitinib was administered daily via oral gavage up to 1000 and 60 m/kg/day to pregnant rats (GDs 6–17) and rabbits (GDs 7–19), respectively. Mean total plasma toxicokinetic parameters in maternal rats and rabbits are summarized in Table 3 with steady-state total plasma concentrations on GDs 17 and 19, respectively, plotted in Figure 7. Reproductive performance and embryo–fetal toxicity parameters are summarized in Table 4.

In maternal rats, mean plasma exposure ($AUC_{0–24}$), C_{max}, and C_{ave} increased with izencitinib dose (Table 3). Systemic exposures on GDs 6 and 17 were generally similar indicating no appreciable accumulation. Similar to the general toxicity studies, plasma izencitinib concentrations in maternal rats were compared with the uncorrected and human plasma protein binding corrected (gray-

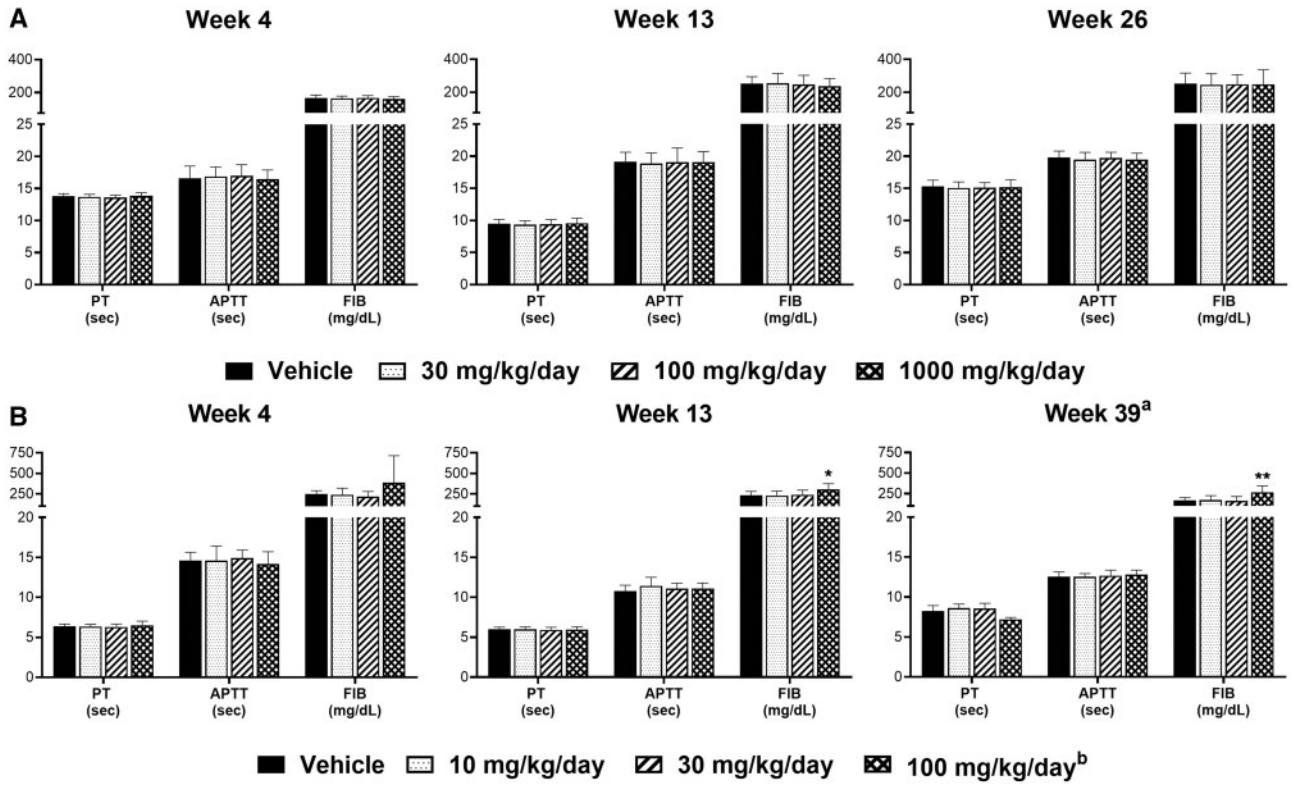

Figure 6. Coagulation parameters following izencitinib administration to rats and dogs. Coagulation parameters following 4, 13, 26 (rats only), or 39 (dogs only) weeks of daily, oral izencitinib administration. Asterisks indicate significant difference from vehicle within species and sex: $^{*}p < .05$, and $^{**}p < .01$. [a]Week 28 values shown for 100 mg/kg/day dose in dogs due to termination of the dose group at week 29; significance with respect to controls at week 28 is shown. [b]In the 4-week study dogs were given 300 mg/kg/day from days 1 to 12 (females) or days 1–13 (males). Due to adverse clinical observations, the high dose of 300 mg/kg/day was then lowered to 100 mg/kg/day.

Table 2. Summary of Rat Mating Performance Following Izencitinib Administration

Dose (mg/kg/day)	Total females paired (*n*)	Females mated (*n*)[a]	Pregnancy rate (%)[b]	Mating index (%)[c]	Fertility index (%)[d]
0	22	22	100.0	100.0	100.0
30	22	22	90.9	100.0	90.9
100	22	21	95.5	95.5	95.5
1000	22	21	90.9	95.5	95.2

Mating performance parameters observed in a fertility and early embryonic development toxicity study following daily, oral administration of izencitinib to male and female rats beginning 14 days prior to mating and continuing up to gestation day 7 in females and up to a minimum of 35 days in males.
[a]Mated = total confirmed mating date and pregnant with no confirmed mating date.
[b]Pregnancy rate = number pregnant/number paired. Restricted to females with confirmed mating date.
[c]Mating Index = mated/number paired.
[d]Fertility Index = number pregnant/mated.

Table 3. Summary of Rat and Rabbit Maternal Izencitinib Plasma Toxicokinetic Parameters

Species	Gestation Day	Dose (mg/kg/day)	Mean C_{max} (ng/ml)	Mean $AUC_{0–24}$ (ng·h/ml)	Mean C_{ave} (ng/ml)
Rat	6	30	1.85	14.7	0.613
		100	5.99	51.6	2.15
		1000	102	389	16.2
	17	30	2.75	15.9	0.663
		100	6.00	69.1	2.88
		1000[a]	161	572	23.8
Rabbit	7	10	7.67	8.77	0.365
		30	40.3	78.8	3.28
		60	305	524	21.8
	19	10	11.4	18.1	0.754
		30	311	448	18.7
		60[a]	860	1840	76.7

Maternal mean total plasma toxicokinetic parameters on gestation days (GDs) 6 and 17 (rats) and GDs 7 and 19 (rabbits) following daily, oral izencitinib administration during the period of organogenesis in EFD toxicity studies. [a] The no observed adverse effect level determined in each study.

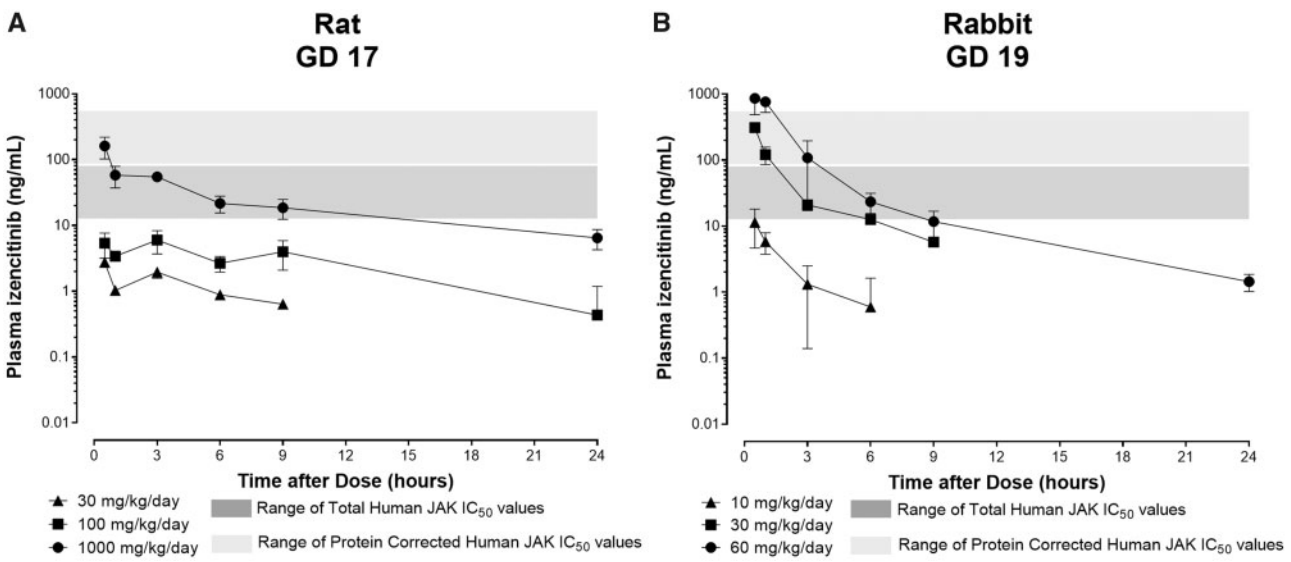

Figure 7. Maternal plasma exposure profiles following daily izencitinib administration in pregnant rats and rabbits. Izencitinib plasma exposure profiles on gestational day (GD) 17 (A, rat) or GD 19 (B, rabbit) following daily, oral administration of izencitinib during the period of organogenesis in embryo-fetal development toxicity studies plotted against uncorrected (12.73–80.31 ng/ml) and human plasma protein binding corrected (87.18–550.1 ng/ml) human cellular Janus kinase 50% inhibitory concentration ranges (Sandborn *et al.*, 2020).

and green-shaded areas, respectively; Figure 7A) human cellular JAK IC_{50} values (Sandborn *et al.*, 2020). Plasma izencitinib concentrations on GD 17 achieved supratherapeutic levels at the 1000 mg/kg/day dose level for 12–15 h (2× plasma C_{ave}:uncorrected JAK IC_{50} ratio; <1× plasma C_{ave}:protein-corrected JAK IC_{50} ratio). Plasma concentrations at 30 and 100 mg/kg/day dose levels were subtherapeutic at all time points assessed (<1× C_{ave}:uncorrect and protein-corrected IC_{50} ratio). Izencitinib was detected (1.18 ng/ml) in the plasma of 1 rat fetus at 3 h postdose at the 1000 mg/kg/day maternal dose level (data not shown). Otherwise, all fetal

Table 4. Summary of Rat and Rabbit Reproductive Performance and Cesarean Section Observations Following Izencitinib Administration

Species	Dose (mg/kg/day)	Total females	Pregnant females	Females with live fetuses	Corpora lutea (mean ± SD)	Implantation sites (mean ± SD)	Preimplantation loss (mean ± SD)	Total resorptions (mean ± SD)
Rat	0	22	21	21	14 ± 2.1	12 ± 2.0	1.0 ± 1.3	0 ± 0.4
	30	22	22	22	14 ± 2.0	14 ± 2.0	1.0 ± 0.9	0 ± 0.6
	100	22	22	22	14 ± 2.3	13 ± 1.8	1.0 ± 1.2	0 ± 0.7
	1000	22	22	22	14 ± 1.7	13 ± 1.9	1.0 ± 1.0	0 ± 1.1
Rabbit	0	22	22	20	10 ± 1.5	10 ± 1.6	1.0 ± 1.0	1 ± 1.6
	10	22	22	20	10 ± 1.7	10 ± 1.6	1.0 ± 0.8	0 ± 0.4
	30	22	21	21	11 ± 2.0	10 ± 1.9	1.0 ± 0.9	0 ± 0.4
	60	22	22	21	11 ± 2.3	10 ± 2.8	1.0 ± 1.5	1 ± 1.6

Embryo–fetal toxicity parameters observed following daily, oral administration of izencitinib to pregnant rats and rabbits during the period of organogenesis on GDs 6–17 and 7–19, respectively.

plasma izencitinib concentrations were below the lower limit of quantitation (<1 ng/ml). Izencitinib was generally well tolerated with minimal variations in maternal body weight gain and food consumption at 1000 mg/kg/day (data not shown). There were no effects on cesarean section parameters (number of corpora lutea, implantation sites, preimplantation loss, or total resorptions; Table 4), and there were no observations of fetal external, visceral, or skeletal malformations across all doses resulting in a NOAEL for rat maternal and EFD toxicity of 1000 mg/kg/day.

In maternal rabbits, mean plasma exposure to izencitinib (AUC_{0-24}) increased greater than the dose increment from 10 to 60 mg/kg/day, with higher exposures on GD 19 compared with GD 7 (Table 3). C_{max} and C_{ave} values also increased greater than the dose increment with higher values observed over time. Plasma izencitinib concentrations on GD19 were compared with both uncorrected and human plasma protein binding corrected (gray- and green-shaded areas, respectively; Figure 7B) human cellular JAK IC_{50} values (Sandborn *et al.*, 2020). Plasma concentrations at the 30 and 60 mg/kg/day dose levels achieved supratherapeutic levels for 6–9 h, corresponding to 1× and 6× plasma C_{ave}:uncorrected JAK IC_{50} ratios, respectively (<1× plasma C_{ave}:protein-corrected JAK IC_{50} ratios for both dose levels). At the 10 mg/kg/day dose level, plasma concentrations remained subtherapeutic at all time points (<1× plasma C_{ave}:uncorrected and protein-corrected JAK IC_{50} ratio). Izencitinib was detectable in the plasma of a single rabbit fetus (1.55 ng/ml) at 3 h postdose at the 60 mg/kg/day maternal dose level (data not shown); otherwise, izencitinib was undetectable in rabbit fetal plasma (<1 ng/ml, limit of quantitation). Izencitinib was well tolerated in rabbits up to 60 mg/kg/day. There were no adverse effects on cesarean section parameters at any dose (Table 4), and no effects on fetal parameters. As such, the NOAEL for maternal and EFD toxicity in rabbits was determined to be 60 mg/kg/day izencitinib.

Izencitinib Clinical Translation

Steady-state total plasma exposures across all doses in each of the nonclinical toxicity studies were compared with human steady-state total plasma exposures (AUC_{0-24} = 55.71 ng·h/ml) achieved following 300 mg izencitinib administration (highest tested dose) in a phase 1 multiple ascending dose study in healthy subjects (Sandborn *et al.*, 2020). These data were used to derive nonclinical:clinical systemic exposure ratios based on the suggestion of izencitinib's highest clinical effect at 300 mg, QD. In the general toxicity studies, the systemic exposures at the NOAEL dose (Figure 8, blue symbols) in each study ranged approximately 6- to 18-fold above the highest clinical exposures achieved, with final systemic exposure margins at the NOAEL of 8-fold in both the 26- and 39-week rat and dog chronic toxicity studies, respectively. Reproductive toxicity exposure margins were even greater with 10- and 33-fold nonclinical:clinical AUC ratios at the NOAEL (Figure 8, blue symbols) in rat and rabbit EFD studies, respectively.

DISCUSSION

The results of the izencitinib nonclinical studies support the concept of the gut-selective design as a method to achieve localized gut exposure while decreasing overall systemic liabilities. Similar approaches with other molecules have been utilized successfully for targets that would not be tolerated systemically such as inhaled bronchodilators (cardiovascular risk), inhaled steroids (immunosuppression and glucose dysregulation), and topical retinol (teratogenicity). Prior work demonstrated that izencitinib is a potent pan-JAK inhibitor that maximizes gut concentrations but limits plasma exposures, thus decreasing the likelihood of systemic safety liabilities (Sandborn *et al.*, 2020). To highlight the differences in exposure between izencitinib and the systemic JAK inhibitor tofacitinib, oral pharmacokinetic data were compared at 10 mg/kg in mice, resulting in colon:plasma AUC ratios of 3883 and 41, for izencitinib and tofacitinib respectively (Sandborn *et al.*, 2020). In a mouse colitis model, izencitinib and tofacitinib achieved similar efficacy at

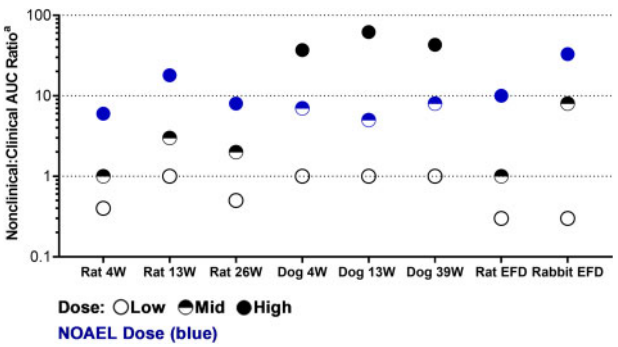

Figure 8. Izencitinib plasma exposure multiples in nonclinical toxicity studies. Ratios of steady-state izencitinib plasma exposures at the no observed adverse effect level dose (blue) in 4-, 13-, 26- (rats only), or 39-week (dogs only) repeat-dose toxicity and rat and rabbit embryo–fetal development toxicity studies versus systemic exposures achieved in a phase 1 multiple ascending dose study in healthy subjects. [a]Exposure ratio based on systemic exposures (area under the curve [AUC]0–24) at 300 mg (AUC0–24 = 55.71 ng·h/ml) tested in a phase 1 multiple ascending dose study in healthy subjects (Sandborn *et al.*, 2020).

1 mg/kg BID and 10 and 15 mg/kg TID, respectively, but only izencitinib spared JAK inhibitor-associated splenic natural killer (NK) cell reductions (Sandborn *et al.*, 2020). Additionally, izencitinib was well tolerated in phase 1 single and multiple ascending dose clinical trials up to 1000 and 300 mg, QD (the highest tested dose), respectively, and up to 270 mg, QD in a phase 1b study in ulcerative colitis patients without the adverse effects commonly observed with systemic JAK inhibition (ie, hematology, creatinine phosphokinase, or lipid level changes; Sandborn *et al.*, 2020). In addition to a favorable safety profile, early indications of izencitinib's clinical effect (ie, improved endoscopy and rectal bleeding scores, decreased serum C-reactive protein) were reported at 80 and 270 mg, QD in a phase 1b study in moderately-to-severely active ulcerative colitis patients (Sandborn *et al.*, 2020). In this work, it is shown that at clinically relevant exposures, the gut-selective design of izencitinib limits JAK inhibitor-associated systemic toxicities in nonclinical safety studies.

Nonclinical studies with other JAK inhibitors have demonstrated a significant immunosuppressive effect with systemic JAK inhibition at subtherapeutic (ie, $<1\times$) nonclinical:clinical AUC margins, which raises concern for secondary effects arising from a lack of immunosurveillance including opportunistic infection and perhaps malignancy (European Medicines Agency, 2012, 2013, 2017a,b, 2019; U.S. Food and Drug Administration, 2011, 2012, 2018, 2019; Pfizer, 2021). Namely, effects consistent with JAK1/3 inhibition have been observed nonclinically, including decreased circulating lymphocytes, neutrophils, B and NK cells, as well as cellular depletion in lymphoid organs and bone marrow (European Medicines Agency, 2012, 2013, 2017a,b; 2019; U.S. Food and Drug Administration, 2011, 2012, 2018, 2019; Gadina *et al.*, 2019; Salas *et al.*, 2020). Decreases in red blood cell parameters (ie, red blood cell counts, hematocrit, hemoglobin) likely driven by JAK2 inhibition have also been reported (European Medicines Agency, 2012, 2013, 2017a, b; 2019; U.S. Food and Drug Administration, 2011, 2012, 2018, 2019; Gadina *et al.*, 2019; Salas *et al.*, 2020). Strong immunosuppressive effects as observed in nonclinical studies with systemically available JAK inhibitors are unsurprising given current knowledge of the role of JAK isoforms in maintaining bone homeostasis and in immune cell differentiation/maintenance in lymphoid tissues (Damerau *et al.*, 2020; Gadina *et al.*, 2019; Ghoreschi *et al.*, 2009; Rochman *et al.*, 2009; Salas *et al.*, 2020; Thomis and Berg, 1997a,b). Though posited as clinically manageable, tolerability for JAK inhibitor-mediated overt immunosuppression in the treatment of inflammatory diseases, unlike neoplastic disorders, is arguably lower and may necessitate clinical dose reductions. Indeed, dose-dependent infections and infestations at $\geq$1 mg, BID were observed in phase 2 studies of tofacitinib in rheumatoid arthritis patients, which coincided with reductions in neutrophils (Fleischmann *et al.*, 2012; Kremer *et al.*, 2009, 2012). Likewise, an increased incidence of herpes zoster and tuberculosis infection was observed in rheumatoid arthritis patients administered baricitinib with rates achieving statistical significance at the 4 mg but not the 2 mg dose (Winthrop *et al.*, 2020). Phase 3 safety data from upadacitinib trials also suggest an increased rate of infection including serious and opportunistic infections and herpes zoster, with higher rates observed at the 30 versus 15 mg dose (Cohen *et al.*, 2020). These results demonstrate further therapeutic benefit may be possible if systemic liabilities can be mitigated such as that proposed with the izencitinib gut-selective approach.

The nonclinical safety studies with izencitinib demonstrate that at higher doses, systemic concentrations can be achieved within the pharmacological range (ie. $1\times$ plasma C_{ave}:uncorrect JAK IC_{50} ratio). Furthermore, at the NOAEL doses (1000 and 30 mg/kg/day in rats and dogs, respectively) in chronic toxicity studies, nonclinical exposures can surpass clinical exposures by 8-fold (nonclinical:clinical systemic AUC margin). Nonetheless, these nonclinical doses and corresponding systemic exposures resulted in minimal primary pharmacology (ie, slight reductions in white and red cell parameters). As expected, when higher doses were administered to dogs ($\geq$100 mg/kg/day izencitinib), supratherapeutic systemic concentrations ($\geq 7\times$ plasma C_{ave}:uncorrected JAK IC_{50} ratio) could be achieved with secondary effects of JAK inhibition (ie, *Demodex spp*. infestation) arising from compromised immunosurveillance. These effects occurred at high nonclinical:clinical systemic AUC margins ($43\times$) in the chronic, repeat-dose dog toxicity study specifically. Observations of reduced immunosurveillance at higher systemic exposures are consistent with dose-limiting opportunistic infections in dogs reported with baricitinib at nonclinical:clinical systemic AUC margins of approximately $31\times$ (compared with reported clinical AUC observed at 2 mg, QD baricitinib); however, microscopic evidence of follicular parasites were also evident at lower AUC margins of approximately $4\times$ (European Medicines Agency, 2017a; U.S. Food and Drug Administration, 2018). Furthermore, baricitinib has been limited to an FDA-approved clinical dose of 2 mg, QD (rather than 4 mg, QD) due to adverse events associated with systemic JAK inhibition (European Medicines Agency, 2017a; U.S. Food and Drug Administration, 2018). Comparing more broadly, determination of the maximum tolerated doses and NOAEL doses for the orally bioavailable JAK inhibitors tofacitinib and ruxolitinib, respectively, was largely driven by significant suppressive effects on the hematopoietic system and resulted in subtherapeutic ($<1\times$) nonclinical:clinical systemic AUC margins (compared with reported clinical AUC observed at 22 mg, QD tofacitinib and 25 mg, BID ruxolitinib; European Medicines Agency, 2012, 2013, 2017b; U.S. Food and Drug Administration, 2011, 2012). Upadacitinib exposures in chronic rat and dog repeat-dose toxicity studies also resulted in somewhat comparable (approximately 2–$13\times$) nonclinical:clinical systemic AUC margins; however, upadacitinib exposures are limited to a greater extent by reproductive and developmental toxicities, described below (European Medicines Agency, 2019; U.S. Food and Drug Administration, 2019).

In addition to immunosuppression, reported elevations in lipids as observed with some systemic JAK inhibitors such as tofacitinib and baricitinib, have drawn concern due to increased risk for MACEs (Choy, 2019; Pfizer, 2021). However, the observed dyslipidemia appears reversible in some cases, and the incidence of MACE may have overlap with underlying cardiovascular risk factors in patients (Choy, 2019; Gadina *et al.*, 2019; Sands *et al.*, 2020). Although dyslipidemia has been described clinically with tofacitinib and baricitinib, no effects on lipid levels were observed at any dose in either the rat or dog repeat-dose toxicity studies conducted with izencitinib (Choy, 2019). Perhaps, the most prominent cardiovascular finding reported with systemic JAK inhibitors relates to a potential prothrombotic risk in patients with underlying cardiovascular risks (Cohen *et al.*, 2020; Gadina *et al.*, 2019). Though this effect is poorly understood to date, it has been postulated that it may be related to JAK2 inhibition and platelet homeostasis as evidenced by elevations in platelet levels (109%–132% of control) observed in rats treated with baricitinib (European Medicines Agency, 2017a). However, the link between JAK inhibition and thromboembolic events has not been causally established

though thrombocytosis has been observed clinically with baricitinib administration (European Medicines Agency, 2017a; Gadina *et al.*, 2019). Evaluation of coagulation parameters in rats and dogs following chronic izencitinib administration produced no significant findings. Reports of nonclinical coagulation data are limited with other JAK inhibitors, though elevations in activated partial thromboplastin time were observed at higher baricitinib doses (≥30 mg/kg/day) in early non-GLP studies in rats (European Medicines Agency, 2017a; U.S. Food and Drug Administration, 2018). However, the predictivity of the standard nonclinical safety coagulation panel as it relates to the thromboembolic events observed clinically with systemic JAK inhibition is unknown. In fact, it is unclear whether rats and dogs are sensitive to such thromboembolic events, perhaps due to as yet unknown species differences or the fact that standard nonclinical safety species do not have underlying cardiovascular risks. In summation, these effects illustrate the fine balance needed to limit the undesired effects of systemic JAK inhibition without limiting the ability to dose sufficiently high to achieve full efficacy.

Reproductive and developmental toxicity studies with systemically available JAK inhibitors have demonstrated risk to female fertility and teratogenicity (Agrawal *et al.*, 2020; European Medicines Agency, 2012, 2013, 2017a,b, 2019, 2020; U.S. Food and Drug Administration, 2011, 2012, 2018, 2019). Fetal findings following maternal JAK inhibitor administration have included weight changes, external and visceral effects, and skeletal malformations such as fused ribs, vertebral anomalies, and misshapen and/or shortened limbs. Such effects coincide with the identified role of the JAK–STAT pathway in bone morphogenesis in knockout animal studies (Damerau *et al.*, 2020). Effects of JAK inhibition on female fertility have been characterized by decreased pregnancy rate and corpora lutea and increased pre- and postimplantation loss and resorptions. Because the manifestation of teratogenicity arises from the dose administered, timing of administration during development, and the ability of the molecule to cross the placental barrier (Cardonick and Iacobucci, 2004), it is expected that an organ-selective molecule that limits maternal systemic exposure would result in a reduced fetal risk. Indeed, izencitinib had no effect on female fertility and no effects on embryonic development in rats up to 1000 mg/kg/day, with a 10× nonclinical:clinical AUC margin. In rabbits, no effects on embryonic development were observed up to 60 mg/kg/day, with a 33× nonclinical:clinical AUC margin. At the highest dose tested in both species, plasma izencitinib concentrations rose above the JAK IC_{50} for at least 9 h (≥2× C_{ave}:uncorrected IC_{50} ratio); however, negligible fetal exposure was observed, highlighting a potential role of the placental barrier in protecting the developing fetus.

Recently, concerns regarding JAK inhibitor effects on male reproductive organs have arisen (European Medicines Agency, 2020). In the rat and dog repeat-dose toxicity studies with filgotinib, microscopic findings in the testis and epididymides were observed at subtherapeutic nonclinical:clinical AUC margins and included germ cell depletion, tubular vacuolation, and sloughed cells (European Medicines Agency, 2020). Negative effects on sperm quantity and quality were similarly noted and functionally manifested in a reduced fertility index in dedicated nonclinical studies, leading to a specific fertility warning for the marketed product (European Medicines Agency, 2020). In contrast, izencitinib had no effect on male or female fertility up 1000 mg/kg/day in rats. Additionally, no effects on organ weights and no microscopic findings were identified in male reproductive tissues in any of the repeat-dose toxicity studies in rats and dogs up to the highest tested izencitinib doses.

CONCLUSION

In conclusion, the gut-selective approach exemplified by izencitinib limited systemic concentrations and resulted in minimal systemic findings, with an 8× nonclinical:clinical systemic AUC exposure margin compared with the highest exposures achieved in a phase 1 clinical study (300 mg, QD). As expected with higher doses, systemic concentrations could be achieved which were sufficient to result in immunosuppression in dogs (≥100 mg/kg/day izencitinib), but at high nonclinical:clinical systemic AUC exposure margins (43×). This reflects the fact that at supratherapeutic systemic concentrations, systemic JAK inhibition was observed. No effects on male or female fertility were observed with izencitinib administration. Additionally, no effects on EFD were observed in rats and rabbits at comparable maternal systemic concentrations (2–6× plasma C_{ave}:uncorrected JAK IC_{50} ratio), resulting in 10× and 33× nonclinical:clinical systemic AUC exposure margins, respectively, compared with the highest exposures achieved in a phase 1 clinical study (300 mg, QD). It is expected that at lower clinical doses under investigations for the treatment of ulcerative colitis and Crohn's disease (20, 80, and 200 mg, QD), nonclinical:clinical margins may be even higher. Together, these data highlight the advantages of organ-selectivity in reducing the dose-limiting systemic safety findings related to on-target JAK pharmacology.

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DECLARATION OF CONFLICTING INTERESTS

P.B., I.B., K.P., G.O., D.B., and E.H. are current employees of Theravance Biopharma US, Inc. and shareholders of Theravance Biopharma, Inc. R.H., M.C., and A.K. are shareholders of Theravance Biopharma, Inc.

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