



# Immunological characterization of HM5023507, an orally active PI3K $\delta/\gamma$ inhibitor

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## Abstract

Phosphoinositide 3-kinases, delta (PI3K $\delta$ ) and gamma (PI3K $\gamma$ ) are enriched in immune cells and regulate the development and function of innate and adaptive immunity. Dual PI3K $\delta/\gamma$  inhibitors are considered high value targets for their potential to treat a variety of immune-mediated diseases, but their discovery has been challenging. Here we describe the preclinical pharmacology of HM5023507, an orally active dual inhibitor of  $\delta/\gamma$  isoforms in immune signaling. HM5023507 inhibited PI3K $\delta$  and PI3K $\gamma$  isoforms with greater than 100-fold selectivity against PI3K $\alpha$  and PI3K $\beta$  in recombinant enzymatic assays and in primary human immune cells with an exquisite selectivity against other targets. HM5023507 attenuated the PI3K $\delta/\gamma$  signaling in human basophils ( $IC_{50}$ : 42/340 nmol/L; selectivity ratio ~1:8). HM5023507 attenuated the activation and function of human B and T cells, Th17 differentiation of CD4 T cells in the blood of healthy donors and rheumatoid arthritis patients, and cytokine and IgG production in human T and B cell cocultures, in vitro. Orally dosed HM5023507 attenuated PI3K  $\delta/\gamma$ -mediated immune signaling in the rat in a dose-related manner. In addition, HM5023507 inhibited semiestablished collagen-induced arthritic inflammation in the rats ( $ED_{50}$  of 0.25mg/kg, p.o. BID or 0.5 mg/kg, QD, AUC: 1422 ng/mL $\cdot$ h), improved histopathology- and micro-computed tomography ( $\mu$ CT)-based indices of joint damage, bone destruction, and attenuated the levels of anti-collagen antibody, with an overall anti-inflammatory profile matching that of a TNF $\alpha$  neutralizing antibody. The PI3K  $\delta/\gamma$  inhibitory profile of HM5023507 and its selectivity make it a useful tool to further delineate immunobiology of dual PI3K  $\delta/\gamma$  targeting.

## KEYWORDS

collagen-induced arthritis, HM5023507, PI3K $\delta/\gamma$  dual inhibitor

**Abbreviations:** Akt, protein kinase B; ATP, adenosine triphosphate; AUC, area under the curve; BCR, B cell receptor; BID, twice a day; C(II), type II collagen; C5a, complement component 5a; CFA, complete Freud's adjuvant; CIA, collagen-induced arthritis; Con-A, concanavalin-A; CXCL1, chemokine (C-X-C motif) ligand 1 (CXCL1); CYP, human cytochrome P450; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; FACS, flow cytometry analysis; FITC, fluorescein isothiocyanate; LC-MS, liquid chromatography-mass spectrometry; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; PEG, polyethylene glycol; PI3K, phosphoinositide 3-kinase; PIP3, phosphatidylinositol (3,4,5) triphosphate; PK, pharmacokinetics; QD/QOD, once daily/once every two days.

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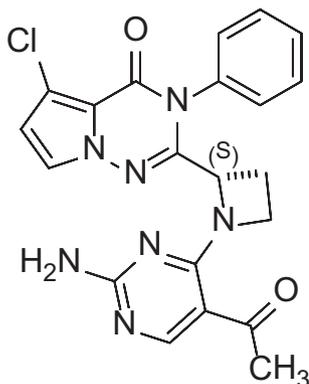
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## 1 | INTRODUCTION

The lipid kinases (PI3K $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) are involved in diverse cellular functions such as cell growth, proliferation, differentiation, motility, survival, and trafficking and have been targeted extensively for drug discovery to treat cancer, autoimmune, and inflammatory diseases.<sup>1-9</sup> These efforts have focused initially on pan-PI3K inhibitors, and subsequently on isoform-selective and dual isoform inhibitors.<sup>8,10-13</sup> Nearly 15 compounds of varying PI3K isoform selectivity have been explored in the clinic culminating in the launch of four drugs to treat different forms of cancers: idelalisib [Zyldig®, a PI3K $\delta$  selective inhibitor],<sup>5</sup> duvelisib [Copiktra®, a dual PI3K $\delta/\gamma$  inhibitor],<sup>14</sup> alpelisib [Piqray®, a PI3K $\alpha$  inhibitor],<sup>15</sup> and copanlisib [Aliqopa®, BAY 80-6946, a dual PI3K $\delta/\alpha$  inhibitor].<sup>16</sup>

Dual inhibitors of PI3K $\delta$  and PI3K $\gamma$  are considered high-value targets both for inflammation/ autoimmunity and oncology.<sup>8,9</sup> Compelling preclinical biology of PI3K $\delta/\gamma$ , initially garnered from gene knockout studies and later substantiated through the use of small-molecule inhibitors,<sup>7,17-26</sup> led to significant investments by the pharmaceutical industry in the last two decades reflected in approximately ~220 patent publications (~80% on PI3K $\delta$  inhibitors, ~15% on PI3K $\gamma$  inhibitors, and ~5% for dual inhibitors).<sup>9</sup>

Duvelisib (also known as IPI-145<sup>13</sup>) is the only dual PI3K $\delta/\gamma$  inhibitor approved to treat relapsed/refractory chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL,<sup>14</sup> at a dose of 25 mg, twice-a-day, associated with dual  $\delta/\gamma$  inhibition. Duvelisib demonstrated compelling activity in various preclinical models of autoimmunity, including arthritis,<sup>13</sup> however, efficacy could not be demonstrated in rheumatoid arthritis clinical trials<sup>27</sup> in the dose range associated with a predominant PI3K $\delta$  inhibition (0.5-5 mg, twice a day, PO). It is speculated that a more balanced PI3K $\delta/\gamma$  dual inhibitor, approaching an ideal ratio of 1:1 with an improved safety profile may be needed to treat chronic autoimmune conditions. Our efforts to identify such inhibitors resulted in the identification of HM5023507 (Figure 1) whose preclinical pharmacology is summarized in this report. The structure-activity relationships and the medicinal chemistry efforts leading up to the identification of HM5023507 are summarized elsewhere.<sup>28</sup>



**FIGURE 1** Chemical structure of HM5023507

## 2 | MATERIALS AND METHODS

### 2.1 | Reagents

HM5023507 was synthesized by Hutchison MediPharma Limited. Details on the source of other reagents can be found in the Supplementary Materials and Methods section. Duvelisib was used as a reference tool and was also synthesized by Hutchison MediPharma.

### 2.2 | Animals

Female Wistar rats (4-6 weeks old) were obtained from Shanghai SLAC Laboratory Animal Co. Ltd and kept under SPF conditions at the animal facility of HMP. Experiments using mice were conducted at Janssen. All animal procedures were approved by the respective institutional animal care and use committees (IACUCs).

### 2.3 | Off-target selectivity profile

The selectivity of HM5023507 was examined at a concentration of 10  $\mu\text{mol/L}$  in a panel of ~50 GPCRs, ion channels and transporters (CEREP panel™).

### 2.4 | Binding activity to recombinant kinases

The kinase selectivity profile of HM5023507 was evaluated using the KINOMESCAN™ assay (DiscoverX), an active site-directed competition binding assay for quantitative measurement of interactions of test compounds with more than 458 recombinant human kinases and disease-relevant mutant variants, at a concentration of 10  $\mu\text{mol/L}$ . KINOME™ assays do not require ATP and thereby report true thermodynamic interaction affinities, as opposed to  $\text{IC}_{50}$  values, which often depend on the ATP concentration used in such assays. In brief, DNA-tagged recombinant kinases are allowed to bind to ligands immobilized on beads in the presence of test compounds or vehicle (dimethyl sulfoxide). Test compounds that bind to kinase active site, sterically or allosterically interfere with the binding of the kinase with bead-immobilized ligand and are removed during a washing step. DNA-tagged kinases retained on the beads are quantitated by PCR amplification.

### 2.5 | Surface plasmon resonance (SPR) binding to PI3K Isoforms

Binding assays using label-free SPR-based technology (BIACORE™) were performed by Biosensor Tools. Histidine-tagged recombinant PI3K proteins were used in SPR assays: PI3K $\alpha$  (heterodimer of full-length p110 $\alpha$  and p85 $\alpha$ ), PI3K $\beta$  (heterodimer of full length p110 $\beta$  and p85 $\alpha$ ), PI3K $\gamma$  (full-length p110 $\gamma$ ), and PI3K $\delta$  (heterodimer of full-length p110 $\delta$  and p85 $\alpha$ ). It should be noted that heterodimer of full

length p110 $\gamma$  with its regulatory subunit p101 with appropriate stability could not be sourced. Binding studies were performed at 25°C using a Biacore S51 optical biosensor equipped with a nickel-charged NiHC1000 sensor chip (Xantec Bioanalytics) and equilibrated with a running buffer (10 mmol/L HEPES, 150 mmol/L NaCl, 5% glycerol, 10 mmol/L MgCl<sub>2</sub>, 1 mmol/L TCEP, 0.02% Tween-20, pH7.3 with 3% dimethyl sulfoxide added after the couple-capture step). The kinases were diluted 1/80 in a coupling buffer (10 mmol/L HEPES, 150 mmol/L NaCl, 5% glycerol, 10 mmol/L MgCl<sub>2</sub>, 1 mmol/L TCEP, 0.02% Tween-20, pH7.3) for couple-capture onto the sensor surface. HM5023507 was tested in triplicate in a dilution series. Response (R) data were analyzed assuming a simple 1:1 interaction model using a local R<sub>max</sub>.

## 2.6 | Binding activity to endogenous kinases in cell lysates

The binding of HM5023507 (1  $\mu$ mol/L) to native kinases was examined via the KiNativ™ assay (ActivX Biosci), based on the use of biotinylated acyl phosphates of ATP (ATP probes) to irreversibly label protein kinases, lipid kinases and heat shock proteins on their conserved lysine residues in the ATP-binding pocket. Covalent modification of kinases by these ATP probes inversely correlates with kinase occupancy by the inhibitors under investigation. Labeled proteins are then subjected to mass spectrometric analysis to define their identity and establish compound occupancy based on the presence or absence of a covalently attached probe. This assay allows characterization of the interaction of test compounds with ~200 kinases, in their native form, in lysates or intact cells isolated from different species. HM5023507 was profiled in cell lysates prepared from the human Jurkat T cell line, as well as from rat, dog, and monkey white blood cells (WBC), as well as intact cells (human PBMC and rat WBC). The assay methodology is summarized in the Supplemental Materials and Methods section.

## 2.7 | PI3K $\alpha$ , PI3K $\beta$ , PI3K $\delta$ , and PI3K $\gamma$ enzymatic assays

The inhibitory effects of HM5023507 on kinase activity of PI3K were determined in enzymatic assays *in vitro* or in a cellular context as per methods described elsewhere<sup>28</sup> and summarized in the Supplemental Materials and Methods Section.

## 2.8 | Anti-IgE-induced basophil activation assay in human whole blood

Basophil activation assay in human whole blood was performed using the FlowCast® basophil activation test according to the manufacturer's instructions (Bulmann Laboratories AG, Schönenbuch, Switzerland).<sup>28</sup> Briefly, healthy donor whole blood was pretreated with HM5023507 for 30 minutes and target immune cells were

stimulated with anti-IgE (for PI3K $\delta$  mediated mechanism), fMLP (for PI3K $\gamma$  mediated mechanism), or IL-3 (as a negative control). The ratio of CD63<sup>+</sup> positive cells in HM5023507-treated blood as compared to the untreated control was used to determine the percentage inhibition. Data were plotted using Prism software and IC<sub>50</sub> values were determined.

## 2.9 | Human immune cell assays

The inhibitory potency of HM5023507 on PI3K $\gamma$  or PI3K $\delta$  signaling was examined in either isolated immune cells, whole blood or under coculture experiments as per methods summarized in the Supplemental Materials and Methods section. Briefly, the effects of HM5023507 on PI3K $\gamma$  signaling were explored by studying IL-8 induced neutrophil shape change.<sup>19,29</sup> The effects on PI3K $\delta$  signaling were studied via anti-IgM-induced B cell function.<sup>30</sup> The effects of dual inhibition of PI3K $\gamma/\delta$  in immune cells were studied via changes in T cell activation, differentiation, Treg function, and T/B coculture assays mimicking the germinal center reaction.

## 2.10 | T and B cell coculture

The assay system (DiscoverRx) consists of a coculture of T/B cells with peripheral blood mononuclear cells stimulated with anti-IgM and a mild T cell stimulation cocktail containing Staphylococcal Enterotoxin B 0.02 ng/mL and Toxic Shock Syndrome Toxin-1 (TSST; 0.02 ng/mL) for 84 hours.<sup>31</sup> Inclusion of a test compound in this assay allows evaluation of its impact on B and T cell activation leading to proliferation, cytotoxicity and the secretion of cytokines including TNF $\alpha$ , IL-6, IL-2, IL-17A, and IL-17F, and natural antibody production (IgG).

## 2.11 | Measurement of immune cell populations in blood from compound dosed rats

Female Wistar rats were orally dosed with HM5023507 dissolved in 20% hydroxypropyl-beta-cyclodextrin (HPCD) or vehicle (HPCD) and blood samples were collected at different time points postdosing. Immune cell populations in the blood samples were measured by a differential blood cell counter (SYSMEX XT2000iiv). The plasma concentrations of HM5023507 were determined by liquid chromatography coupled with triple quadrupole mass spectrometer (LC-MS/MS) method with the lower limit of quantification (LLOQ) of 1 ng/mL.

## 2.12 | Con-A-induced cytokine production in rats

Female Wistar rats were orally dosed with HM5023507 dissolved in HPCD or vehicle (HPCD), given a Con-A (30 mg/kg, iv, Sigma-Aldrich)

challenge 0.5 hour or 6.5 hour after oral dosing and euthanized for blood collection 1.5 hour post-Con-A challenge. Serum and plasma samples were respectively processed for quantitation of IFN $\gamma$  (R&D systems) or HM5023507.

### 2.13 | Anti-IgD-induced B cell activation in rat whole blood

Female Wistar rats were orally dosed with HM5023507 dissolved in HPCD or vehicle (HPCD). Blood samples were collected under anesthesia at the designed time points postdose and stimulated with anti-IgD at 37°C overnight. After red blood cell (RBC) lysis, leukocytes were harvested, and B cells were stained with anti-B220 and anti-CD86 antibodies (both from eBioscience) for flow cytometry analysis.<sup>28</sup> Activated B cells were gated based on B220 and CD86 signal. The plasma concentrations of HM5023507 were analyzed by LC-MS/MS methods.

### 2.14 | Chemokine-driven neutrophil migration into rat subcutaneous air pouch

The assay methodology was adopted from Winkler et al.<sup>13</sup> The backs of the female Wistar rats were shaved and injected subcutaneously with sterile air (20 mL on day 0, and 10 mL of on day 3). On day 6, rats were orally dosed with HM5023507 dissolved in HPCD or vehicle (HPCD), and 30 minutes later received intra-pouch injections of recombinant rat CXCL1 (2  $\mu$ g/rat, Peprotech, delivered as solution in 0.5% carboxymethyl cellulose [CMC], 1 mL/rat) or 0.5% CMC, and sacrificed at 4 hours after CXCL1 or vehicle injection. The cells in the air pouches were harvested by injecting 5 mL of PBS containing EDTA and counted by Cellometer Auto T4, and then stained with anti-granulocyte-PE antibody for flow cytometry analysis. The results were reported as the number of neutrophils per cavity.

### 2.15 | Type II collagen-induced arthritis in rats

CIA was induced as per methods outlined in Jia et al.<sup>28</sup> Briefly, female Wistar rats were anesthetized with isoflurane, and 200  $\mu$ g of bovine type II collagen (Chondrex, Redmond, WA) in Freund's incomplete adjuvant (Sigma-Aldrich) was injected intradermally at the base of the tail on days 0 and 6. HM5023507 dissolved in HPCD or vehicle (HPCD) were dosed orally from day 10 onwards. YiSaiPu® (a TNF $\alpha$  Fc fusion protein; Shanghai CP Guojian; 10 mg/kg, twice a week) was administered as an intraperitoneal injection. The hind paw volume was measured with a plethysmometer daily, and the area under the paw volume vs time course curves was calculated. Satellite groups of animals were used to collect blood samples for at selected time points for the determination of plasma levels of HM5023507.

On day 16, the animals were euthanized, sera and paw samples were collected. The paws were decalcified in acetic acid, fixed in 10% buffered formalin, sectioned, and stained with hematoxylin and eosin. Histopathology assessments were made according to a modified Mankin scoring scale which comprises of bone resorption (scale 0-5), cartilage damage (scale 0-6), cartilage cell damage (scale 0-3), tissue inflammation (scale 0-5), and synovial inflammation (scale 0-4). All the scores were totaled as a composite response.

The frozen hind paws without fixing were processed for x-ray computed tomography (CT) imaging. Each paw was placed inside the preclinical CT scanner (Siemens Inveon) and scanned using an x-ray source (80 kVp, 500  $\mu$ A), and a 2048  $\times$  3072 CCD detector, with a 2  $\times$  2 binning. A total of 720 projections were acquired around 360°. Images were reconstructed using a modified Feldkamp algorithm into 1024  $\times$  1024  $\times$  1786 voxel images of spatial resolution 27  $\mu$ m.

The images were analyzed by measuring the mean bone density of the sample (in Hounsfield Units, HU). The surface roughness was determined by measuring the depth of bone destruction and pitting from a plane fit to the local surface. A histogram of surface depths was analyzed by measuring the shift of the roughness distribution to higher depths with increasing disease severity. The ratio of the area under the curve (AUC) in the upper half of the distribution to that in the lower half gave a quantitative measure of this roughness shift.

## 3 | RESULTS

The results are organized into the following sections: (a) PI3K  $\delta\gamma$  dual inhibitory activity, (b) effects on immune cell function *in vitro*, (c) selectivity against off-targets, (d) characterization of inhibition of PI3K  $\delta\gamma$  *in vivo*, (e) impact on immune cell trafficking *in vivo*, and (f) characterization of anti-inflammatory activity *in vivo*.

### 3.1 | HM5023507 is a Potent and Selective PI3K $\delta\gamma$ Dual Inhibitor

In *in vitro* biochemical assays, HM5023507 potently inhibited the kinase activity of both PI3K $\gamma$  and PI3K $\delta$  (Table 1; IC<sub>50</sub> values of 4 and 5 nmol/L, respectively) with selectivity vs other isoforms. The selectivity ratio among the 4 isoforms for HM5023507, with its PI3K $\delta$  IC<sub>50</sub> value [mean] equated to 1, was 1( $\delta$ ):1( $\gamma$ ):148( $\beta$ ):990( $\alpha$ ).

In the SPR assay, the equilibrium dissociation constants (K<sub>D</sub>) for human PI3K $\gamma$ , PI3K $\delta$ , PI3K $\beta$ , and PI3K $\alpha$  were in the order of 1:4:1076:4067 indicating that the rank order of affinity is PI3K $\gamma$  > PI3K $\delta$  » PI3K $\alpha$ /PI3K $\beta$  (Table 2).

The kinome selectivity of HM5023507 was examined in a panel of 458 kinases (395 nonmutated kinases + 63 mutated human kinases), at a test concentration of 10  $\mu$ mol/L (~200 $\times$  times over the IC<sub>50</sub> values for PI3K $\gamma$  and PI3K $\delta$  in biochemical assays). At this concentration, the

**TABLE 1** HM5023507 is a potent and selective inhibitor of PI3K $\gamma$ / $\delta$  kinase activity

PI3K	Protein	IC <sub>50</sub> (nmol/L) <sup>a</sup>
PI3K $\gamma$	p110 $\gamma$	4 ± 2
PI3K $\delta$	p110 $\delta$ /p85 $\alpha$	5 ± 3
PI3K $\beta$	p110 $\beta$ /p85 $\alpha$	590 ± 250
PI3K $\alpha$	p110 $\alpha$ /p85 $\alpha$	3960 ± 570

<sup>a</sup>Mean ± SD (n = 3). The experimental control duvelisib inhibited PI3K isoforms with average IC<sub>50</sub> values (nmol/L) of 140 (PI3K $\alpha$ ), 10 (PI3K $\beta$ ), 2 (PI3K $\gamma$ ), and 1 (PI3K $\delta$ ), respectively (n = 3-4 experiments).

**TABLE 2** HM5023507 selectively binds to PI3K $\gamma$  and PI3K $\delta$  with high affinity in surface plasmon resonance assays

PI3K	HM5023507 <sup>a</sup>		
	k <sub>on</sub> (M <sup>-1</sup> s <sup>-1</sup> )	k <sub>off</sub> (S <sup>-1</sup> )	K <sub>D</sub> (nmol/L)
PI3K $\gamma$	3.71 × 10 <sup>5</sup>	2.20 × 10 <sup>-4</sup>	0.60
PI3K $\delta$	1.74 × 10 <sup>6</sup>	4.21 × 10 <sup>-3</sup>	2.43
PI3K $\beta$	4.1 × 10 <sup>5</sup>	0.27	640
PI3K $\alpha$	9.0 × 10 <sup>4</sup>	0.22	2420

<sup>a</sup>Mean of triplicate measurements from two experiments. The K<sub>D</sub> values (nmol/L) for the experimental control duvelisib against PI3K $\gamma$ ,  $\delta$ ,  $\beta$ , and  $\alpha$  isoforms were 2.12, 0.12, 6.9, and 245, respectively. The SD values were <10% of mean.

HM5023507 showed the highest affinity binding for PI3K $\gamma$ , PI3K $\delta$ , and PI3K $\beta$  with minimal affinity for PI3K $\alpha$  (Supplementary Section, Table S1). In addition, interaction with PI3K $\gamma$ 2G, a Class II PI3K was also observed. In additional assays, preferential interaction of HM5023507 to class I PI3K vs class II PI3K was demonstrated (see below).

The Kinativ™ platform was used to assess binding interactions of HM5023507 (1  $\mu$ mol/L) with native cellular kinases in lysates from Jurkat T cells, and rat dog and cynomolgus leukocytes. HM5023507 showed  $\geq$ 85% binding to endogenous PI3K $\gamma$  and PI3K $\delta$  in cell lysates from the different species evaluated (Supplementary Section, Table S2). The binding affinity to PI3K $\beta$  varied somewhat by species, with low interaction in rat WBC lysates (46%), moderate in dog and monkey WBC lysates (78% and 70%, respectively), and significant binding in human Jurkat cell lysates (87%). HM5023507 did not bind to PI3K $\alpha$  in Jurkat lysates, and its effect on PI3K $\alpha$  in lysates from other species could not be evaluated since the PI3K $\alpha$  signals were below the detection limits of the assay. HM5023507 selectively bound to PI3K $\gamma$  and PI3K $\delta$ , without any detectable binding activities with ~200 other kinases present in cell lysates. These results indicate that HM5023507 is an inhibitor of native PI3K $\gamma$  and PI3K $\delta$  kinases from human, rat, dog, and monkey in their native cellular milieu. Among the panel of native kinases examined, HM5023507 showed minimal interactions with Class II and III PI3Ks demonstrating its PI3K class selectivity.

The binding of HM5023507 to native kinases in intact human PBMC and rat leukocytes was also explored (Supplementary Section, Table S3). HM5023507 potentially bound to both PI3K $\gamma$

and PI3K $\delta$  in human PBMC (IC<sub>50</sub> values of ~100 and 40 nmol/L, respectively) with selectivity against PI3K $\beta$  (IC<sub>50</sub> > 1  $\mu$ mol/L). HM5023507 bound to rat PI3K $\delta$  (IC<sub>50</sub> of 40-60 nmol/L) with modest inhibition of rat PI3K $\gamma$  (IC<sub>50</sub> > 1  $\mu$ mol/L). The latter result is in contrast to ~95% binding inhibition of the same target kinase in rat leukocyte lysate experiment. More convincingly, the inhibitory effects of HM5023507 on PI3K $\gamma$  signaling in vitro and in vivo were established through attenuation of cellular pAKT measurements and neutrophil shape change, and neutrophil chemotaxis, respectively (vide infra).

Measurements of phosphorylated AKT were used to determine the functional consequences of kinase inhibition in the cellular milieu. C5a and  $\alpha$ -IgM were used to activate PI3K $\gamma$  and PI3K $\delta$  pathways in murine macrophage (RAW) cell line and in a human B cell line (RAMOS), respectively. Constitutive pAKT levels in a prostate cancer cell line (PC-3) were used as a measure of PI3K $\alpha$ / $\beta$  signaling. HM5023507 potentially inhibited the kinase activity of PI3K $\gamma$  and PI3K $\delta$  with overlapping IC<sub>50</sub> values (10-25 nmol/L) with selectivity over the PI3K $\beta$  isoform (IC<sub>50</sub> ~ 3500 nmol/L; Table 3). The selectivity ratio among the 3 isoforms for HM5023507, with its PI3K $\delta$  IC<sub>50</sub> value [mean], is equated to 1, was 1( $\delta$ ):0.5( $\gamma$ ): 151 ( $\beta$ ).

### 3.2 | Effects of HM5023507 on immune function

The effects of HM5023507 on immune function were examined using diverse primary human immune cells (T and B cells, and neutrophils and basophils). In addition, the effects of the compound were also examined in rat and human whole blood, a complex biological matrix.

### 3.3 | Activation and differentiation of human T cells, and human Treg Function

Both PI3K $\gamma$  and PI3K $\delta$  have been suggested to play a role in T cell activation.<sup>23,32-35</sup> HM5023507 attenuated anti-CD3/anti-CD28-induced human T cell activation, with a better potency in the blockade of IL-2 production than in proliferation response, as indicated by its IC<sub>50</sub> values of 9 and 120 nmol/L (mean value), respectively (Supplementary Section, Table S4). T cell differentiation into

**TABLE 3** Selectivity of HM5023507 in blocking PI3K $\gamma$ - and PI3K $\delta$ -mediated cellular AKT phosphorylation

PI3K	Assay	HM5023507 IC <sub>50</sub> (nmol/L) <sup>a</sup>
PI3K $\gamma$	C5a-induced pAKT in RAW cell line	11 ± 5 (n = 3)
PI3K $\delta$	$\alpha$ IgM-induced pAKT in RAMOS cell line	25 ± 9 (n = 3)
PI3K $\beta$ / $\alpha$	pAKT in PC3 cell line	3780 ± 600 (n = 3)

<sup>a</sup>Mean ± SD. The positive control duvelisib inhibited PI3K $\gamma$ ,  $\delta$ , and  $\beta$ / $\alpha$  isoforms with average IC<sub>50</sub> values of 29, 0.6, and 52 nmol/L, respectively (n = 3-4 experiments).

different effector cell types including Th1, Th2, Th17, and Treg cells was also reduced by HM5023507 with mean  $IC_{50}$  values of 48, 18, 63, and 130 nmol/L, respectively.

Th17 cells and cytokines derived from them have been implicated in the pathology of several autoimmune diseases including rheumatoid arthritis. The potential modulatory effect of HM5023507 on Th17 cell differentiation in rheumatoid arthritis patients was evaluated *ex vivo* on and compared to that from healthy donors. Differentiation of Th17 cells in the whole blood reflected in increased production of IL-17A, and cytokines such as IL-22 and IFN $\gamma$ . HM5023507 was effective at inhibiting the generation of these cytokines with a similar potency both in healthy and arthritic blood (Table 4).

The CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells (Tregs) contribute to the maintenance of peripheral tolerance by inhibiting the expansion and function of effector T cells. The role of PI3K signaling in Tregs is controversial and context dependent.<sup>34,36</sup> As HM5023507 attenuated human T cell activation and differentiation to different effector cell types including Treg cells, the potential impact of this compound on human Treg suppressive function were further explored in coculture experiments. Since the inhibition of PI3Ks by HM5023507 is reversible, Tregs (CD4<sup>+</sup> CD25<sup>+</sup>) from donor blood were cultured with autologous CD4 T responder cells with or without HM5023507 and stimulated with anti-CD3/CD28 antibodies. Tregs and responder cells differed in their proliferative response to anti-CD3/CD28 stimulation and to HM5023507. While the responder T cells proliferated robustly, Tregs showed a weak proliferative response to anti-CD3/CD28 stimulation. HM5023507 did not impact proliferation or survival of Tregs, but potently inhibited proliferation of responding T cells. In coculture experiments (responder cell: Treg ratio of 1:2), Tregs alone suppressed anti-CD3/CD28-induced proliferation response of responder cells by >90%.

In coculture experiments (responder cell: Treg ratio of 1:2), the inclusion of HM5023507 led to a modest suppression of the proliferative response of responding cells vs coculture control (Supplementary Section, Figure S1). If HM5023507 were to alter Treg suppressive function, then the suppressive effects of Tregs on the proliferative response of T effector cells would be expected to significantly be reduced. The results suggest that

**TABLE 4** HM5023507 is a potent inhibitor of Th17 differentiation in blood samples from rheumatoid arthritis patients and healthy donors

Th17 differentiation in whole blood	HM5023507 $IC_{50}$ (nmol/L) <sup>a</sup>	
	Healthy donor blood	RA patient blood
IL-17A	110 ± 36 (n = 5)	88 ± 71 (n = 14)
IFN $\gamma$	70 ± 44 (n = 5)	66 ± 52 (n = 12)
IL-22	130 ± 46 (n = 5)	73 ± 64 (n = 9)

<sup>a</sup>Mean ± SD. The positive control duvelisib attenuated IL-17A, IFN $\gamma$ , and IL-22 cytokine production in healthy human donors with  $IC_{50}$  values of 50, 41, and 170 nmol/L, respectively (n = 4 donors; average). The corresponding  $IC_{50}$  values in RA patient derived blood were 69, 24, and 57 nmol/L, respectively (n = 8-15 donors; average).

HM5023507 does not markedly affect the Treg function, an interpretation that is consistent with a selective sparing of human Tregs relative to CD4/CD8 T cells by pharmacological inhibitors of PI3K pathways.<sup>36</sup>

### 3.4 | Activation isolated human B cells, and B cells in human and rat blood

PI3K $\delta$  is known to play a critical role in B cell activation.<sup>3,23,24,30</sup> Consistent with its binding and inhibitory activities on PI3K $\delta$ , HM5023507 inhibited anti-IgM-induced activation as measured by upregulation of CD69 and IL-6 production from purified human B cells ( $IC_{50}$  values <10 nmol/L), inhibited anti-IgM-induced B cell activation in human PBMC and whole blood with an average  $IC_{50}$  values of 42 and 63 nmol/L, respectively. HM5023507 also attenuated B cell activation in rat whole blood with an average  $IC_{50}$  of 44 nmol/L (Table 5), showing comparable inhibitory potencies in rat and human whole blood.

### 3.5 | Human neutrophil activation

Activation of neutrophil IL-8 receptor by IL-8 triggers a shape change, a surrogate marker for the ensuing chemotactic response. PI3K $\gamma$  has been implicated in regulating chemokine GPCR signaling and chemotaxis.<sup>23,25,26,37</sup> In accordance with this mechanism, HM5023507 inhibited IL-8 induced shape changes of purified human neutrophils with an average  $IC_{50}$  value of 63 nmol/L (Supplementary Section, Table S5), and in whole blood, the inhibition was demonstrable at a higher concentration (average  $IC_{50}$  1080 nmol/L).

### 3.6 | Human blood basophil activation

Basophil activation plays a key role in allergic inflammation. Activation of basophils via IgE binding to the IgE high-affinity receptor or fMLP binding to the formyl peptide receptor 1 (FPR1) respectively involves PI3K $\delta$  or PI3K $\gamma$  signaling.<sup>23,26,38</sup> As such, the basophil activation assay in human blood provides an opportunity to define PI3K $\delta$  or PI3K $\gamma$  inhibitory ratio in the same cell. HM5023507 inhibited IgE- or fMLP-induced upregulation of CD63 on activated human basophils in blood with average  $IC_{50}$  values of 42 and 337 nmol/L, respectively, suggesting an isoform selectivity of ~ 1:8 (PI3K $\delta$  over PI3K $\gamma$ ) (Supplementary Section, Table S5).

### 3.7 | Human T: B CoCultures

HM5023507 was further evaluated in the BioMAP™ profiling platform (DiscoverRx) on human B and T cell cocultures. In initial studies, HM5023507 was examined a broader panel of BioMAP™ assays that included both immune and nonimmune cells (eg. endothelial cells and fibroblasts). Subsequent investigations focused on most

**TABLE 5** HM5023507 is a potent inhibitor of B cell activation

Species	Human B cell response	Cellular readout	HM5023507 IC <sub>50</sub> (nmol/L) <sup>a</sup>
Human	Anti-IgM-induced activation of purified B cells	B cell proliferation	4 ± 5 (n = 3)
	Anti-IgM-induced B cell activation in PBMC	CD69 upregulation	42 ± 3 (n = 3)
	Anti-IgM-induced B cell activation in whole blood	CD69 upregulation	63 ± 37 (n = 5)
Rat	Anti-IgD-induced B cell activation in whole blood	CD86 upregulation	44 ± 15 (n = 3)

<sup>a</sup>Mean ± SD. The positive control, duvelisib attenuated B cell function with IC<sub>50</sub> values 0.3 nmol/L (human purified B cells), 1 nmol/L (human PBMC) and 13 nmol/L (human whole blood), and 2 nmol/L (rat blood), respectively (average of 3-5 experiments).

responsive system, that is, T:B assay that mimics T cell-dependent B cell activation and class switching, as would occur in the germinal centers of peripheral lymphoid organs, leading to B cell proliferation and antibody production. T cell cytokines such as IL-2, IL-17A, and IL-17F, and B cell cytokine IL-6, and potentially derived from both T and B cells. HM5023507 attenuated the B cell response in a concentration-dependent manner, with a robust inhibition of IgG production, accompanied by modest attenuation of B cell proliferation (Figure 2). HM5023507 attenuated the production of IL-17A and IL-17F, with a modest impact on TNF $\alpha$  and IL-2. The lack of effect of HM5023507 on IL-2 production could be related to the time point of sample collection (~85-hr poststimulation), at which time, most of the IL-2 generated would have been consumed by the proliferating T cells.

### 3.8 | Off-target activity

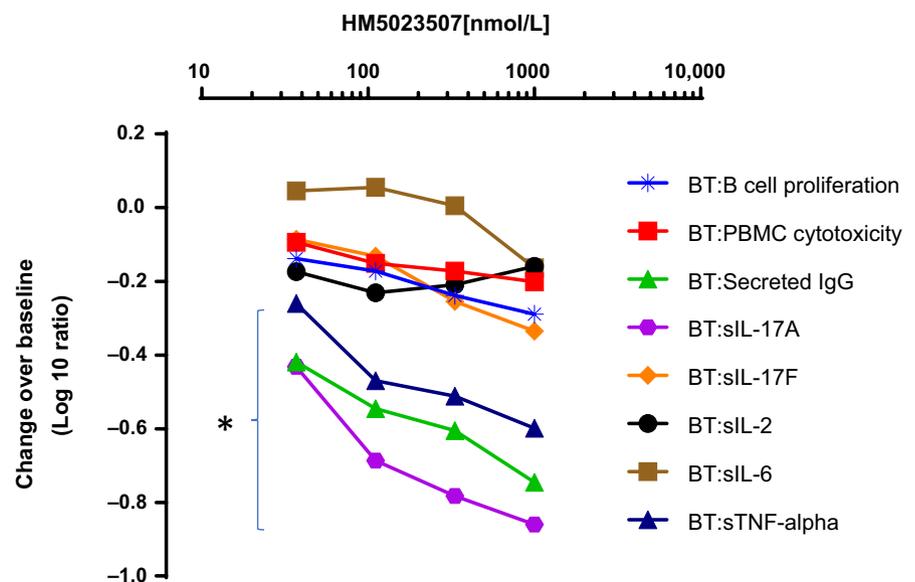
Potential off-target interactions of HM5023507 were investigated in a broad panel of 50 GPCR and ion-channel targets at a test concentration of 10  $\mu$ mol/L (Supplementary Section, Table S6). Except for an increased binding of <sup>3</sup>H-TBPS to GABA binding sites in rat

brain (cortex) homogenates by 152%, there were no other interactions that reached  $\geq$ 30% of the control response. HM5023507 inhibited hERG K channel activity measured in an electrophysiological assay with IC<sub>50</sub> values ranging from 2.3 to 2.9  $\mu$ mol/L. HM5023507 at a test concentration of 3  $\mu$ mol/L showed <10% inhibition against the following ion channels: (L-type calcium channels (hCav1.2.), T-type calcium channels (hCav3.2, hHCN2 channels, hHCN4 channels, hKir2.1 potassium channels, hKir3.1/hKir3.4 potassium channels, Kir6.2/SUR2A potassium channel, hKv1.5 potassium, hKv4.3 potassium channels hKvLQT1/hminK and hNav1.5 sodium channels (SCN5A gene expressed in CHO cells (data not shown).

### 3.9 | Pharmacokinetics of HM5023507 in wistar rats and plasma protein binding

The pharmacokinetic parameters for HM5023507 in female Wistar rats are as follows (mean ± SD, n = 3; iv/po doses of 2.5 and 10 mg/kg, respectively<sup>28</sup>; terminal half-life- 5.1 ± 0.6 hr, plasma clearance- 3.3 ± 0.1 mL/min/kg, Vss: 1.2 ± 0.1 L/kg, and oral bioavailability (%F) - 104 ± 18.

**FIGURE 2** HM5023507 modulates T/B cell signaling under coculture conditions that mimic the germinal center reaction (BioMap phenotypic screening). Changes in measured mediators were compared to baseline response defined by the vehicle. Changes in IL-17A, secreted IgG and TNF $\alpha$  were considered significant ( $P < .05$ ) at all concentrations of HM5023507 (as per statistical analysis methods described in Melton et al.<sup>31</sup>



Plasma Protein Binding of HM5023507 was determined using an equilibrium dialysis method in several preclinical species, including humans. The binding of HM5023507 to rat plasma proteins indicate a free fraction of 9%-12% in the concentration range of 0.5-25  $\mu\text{mol/L}$ . The binding of HM5023507 to human plasma proteins indicate a free fraction of 5.9%-17.5% in the concentration range of 0.5-25  $\mu\text{mol/L}$ . Blood to plasma concentration ratio in several species including human and rat blood was also determined and the partitioning approaches 1.

### 3.10 | Inhibition of PI3K $\delta$ or PI3K $\gamma$ signaling in vivo

The inhibitory effects of HM5023507 on PI3K $\delta$  or PI3K $\gamma$  isoforms by HM5023507 were characterized by monitoring changes in B cell activation or modulation of KC induced neutrophil influx into a subcutaneous air pouch, respectively.

Orally administered HM5023507 attenuated rat B cell activation as measured by suppression of IgD-induced B cell activation reflected in the expression of CD86 surface marker in a dose-related manner. The ED<sub>50</sub> dose ranged from 0.08 to 0.14 mg/kg, p.o. The associated plasma levels were 48-134 nmol/L (Figure 3). A single dose of 1 mg/kg provided >90% inhibition of B cell activation for up to 8 hours with inhibition of ~40%, maintained for up to 24 hours (Figure 3). In a follow-up study, a higher dose of HM5023507 (3 mg/kg, p.o., dosed twice, t = 0 and t = 8 hr) led to >90% suppression of B cell activation for up to 24 hours.

Instillation of CXCL1 into the rat subcutaneous air pouch induced robust neutrophil chemotaxis and this response is known to involve PI3K $\gamma$  signaling.<sup>13,23,37,39</sup> Orally administered HM5023507 attenuated the neutrophil chemotaxis in a dose-related manner (Figure 4), with a clear trend for reduction seen at a dose of 3 mg/kg (p.o.) and a reduction to baseline levels at 10 mg/kg ( $P < .05$ ).

The functional consequences downstream of dual PI3K $\delta/\gamma$  inhibition were assessed via (a) kinetics of lymphocyte/leucocyte in circulation, (b) inhibition of concanavalin-A (Con-A)-induced IFN $\gamma$  changes, and (c) the inhibition of collagen-induced arthritis.

### 3.11 | Immune cell trafficking

Physiological immune surveillance is ensured by continuous trafficking of immune cells throughout the body and recruitment to specific tissue compartments, such as secondary lymphoid organs, for mounting an immune response upon encounter with and recognition of antigens. PI3Ks regulate several aspects of migration such as gradient sensing via the regulation of chemokine signaling and cell motility.<sup>7,8,19,23,29,37,39</sup> Oral administration of HM5023507 led to rapid, dose- and time-dependent increases in circulating leukocytes, with modest increases in circulating neutrophils (Supplementary Section, Figure S2). These changes reflect disruption of chemokine/chemotactic signaling in lymphoid organs by HM5023507 leading to the emigration of immune cells into the circulation.

### 3.12 | Anti-inflammatory activity of HM5023507 in vivo

The anti-inflammatory activity of HM5023507 was examined using concanavalin-A-induced cytokine response and a rat collagen-induced arthritis.

### 3.13 | Concanavalin-A-induced cytokine response

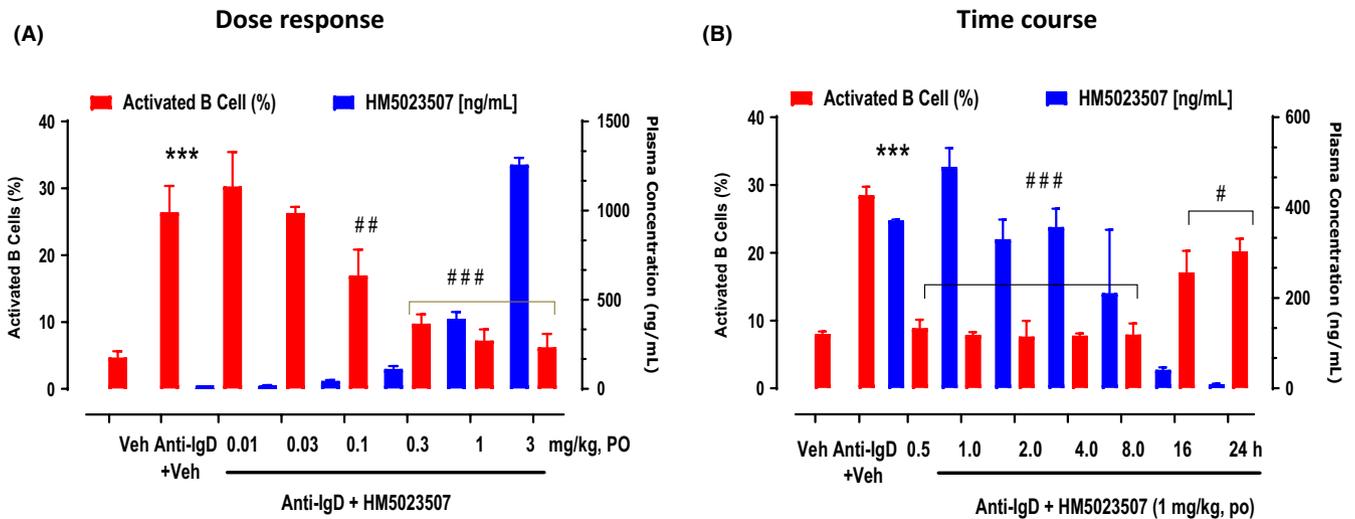
Injection of concanavalin-A (Con-A), a plant lectin and a known T cell mitogen, induces inflammatory cytokines such as IFN $\gamma$ , IL-4, and TNF $\alpha$  in rats and mice. A clear role of PI3K $\gamma$  signaling in Con-A-induced cytokine response was established by significantly lower cytokine response to Con-A treatment in mice harboring an inactive PI3K $\gamma$  kinase vs wild-type mice (Supplemental Figure S3). In rats, Con-A injection increased IFN $\gamma$  levels by 30- to 60-fold over the baseline, and orally dosed HM5023507 attenuated these changes in a dose- and exposure-related manner. At a dose of 10 mg/kg (p.o.), >80% inhibition of plasma IFN $\gamma$  was observed for up to 8 hours after dosing (Figure 5).

### 3.14 | Collagen-induced arthritis

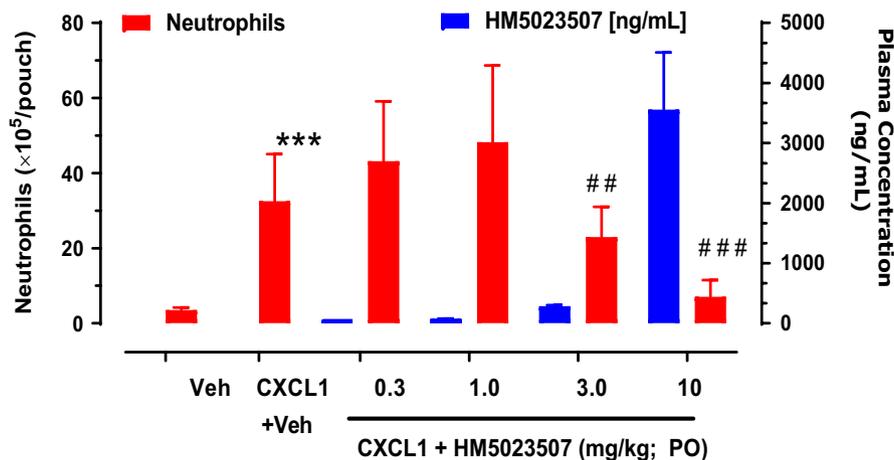
The anti-inflammatory activity of HM5023507 was examined in three separate collagen-induced arthritis experiments in rats. In the first experiment, HM5023507 was examined over a wide dose range under the BID (twice daily, at approximately 8-9 hours between AM/PM doses) regimen, as well as a single dose delivered once-daily (QD). Two subsequent experiments were designed to confirm the key findings from the first experiment and extend them under QD and once every other day (QOD) regimens. A TNF $\alpha$  inhibitor (TNF $\alpha$  receptor Fc fusion protein; a biosimilar to Etanercept; YiSaiPu®), administered under QOD regimen, was used as an experimental control. Representative data are shown in Figure 6A/B and Supplemental Figure S4. The administration of HM5023507 or YiSaiPu® was initiated in a semitherapeutic paradigm in animals with established signs of paw inflammation.

### 3.15 | Paw inflammation

HM5023507 attenuated the hind paw inflammation in a dose-dependent manner (Figure 6, Panel A) with ED<sub>50</sub> values in the range of 0.4-0.5 mg kg<sup>-1</sup> d<sup>-1</sup>. Consistent with similar plasma exposure, the time effect curves of 0.3/3 mg/kg (BID) or 0.6/6 mg/kg (QD) of HM5023507 on paw inflammation were also superimposable. It is noteworthy that anti-inflammatory profile of HM5023507, inferred from the magnitude of reduction of paw inflammation, was comparable to that seen with a biologic agent, YiSaiPu®.



**FIGURE 3** HM5023507 (p.o.) attenuates B cell activation in female Wistar rats. Dose-related effects were defined in blood samples collected 2-h postoral dose of HM5023507 (0.01-3 mg/kg) or vehicle (Panel A). The duration of inhibition was defined by collection of blood at various times after an oral dose of HM5023507 (1 mg/kg, Panel B). Blood samples from vehicle dosed groups without the IgD stimulation and with IgD stimulation were used to define the lowest and highest level of B cell activation, respectively. Data represent mean  $\pm$  SEM ( $n = 3$  rats/group) and were analyzed via one-way ANOVA, followed by Dunnett's test. \*\*\* $P < .001$  vs Veh; ## $P < .01$ , ### $P < .001$  vs Veh + anti-IgD. In a separate study, vehicle or HM5023507 (3 mg/kg, po; two doses given at  $t = 0$  and 8 h) and blood samples were collected for IgD stimulation at 24 h. The inhibition of B cell activation was  $90 \pm 10\%$  (mean  $\pm$  SD;  $n = 3-4$  rats). The plasma levels HM5023507 (ng/mL; mean  $\pm$  SD,  $n = 3$ ) were  $150 \pm 35$ . The experimental control, duvelisib attenuated B cell activation (measured 2 hr post oral dose) with an estimated ED50 of 0.09 mg/kg (data not shown)

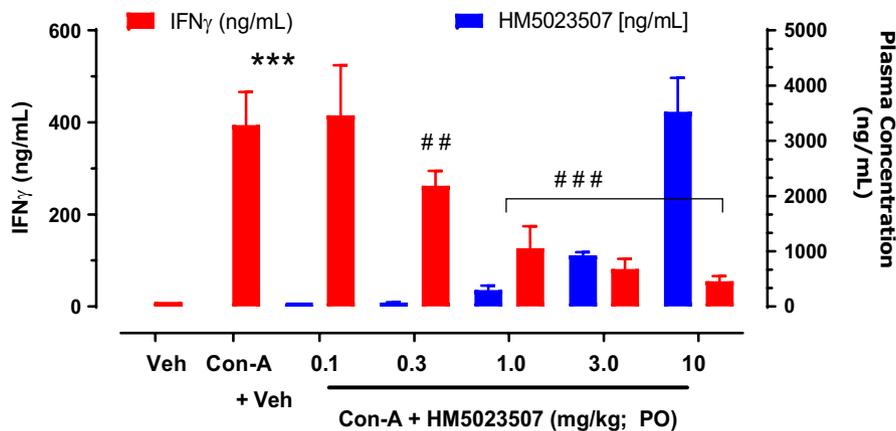


**FIGURE 4** HM5023507 (p.o.) attenuates CXCL1 induced neutrophil influx in female Wistar rats. Rats with preestablished air pouches were orally dosed with HM5023507 or vehicle ( $t = 0$  hr), injected with 1 ml of recombinant rat CXCL1 (2  $\mu$ g/rat pouch) or 1 mL of 0.5% carboxymethyl cellulose (CMC) in PBS at  $t = 0.5$  hr, and euthanized at  $t = 4$  hr. Air pouch fluids were processed to enumerate neutrophils. Data represent mean  $\pm$  SEM ( $n = 8$  rats/group) and were analyzed by one-way ANOVA followed by Dunnett's test. \*\*\* $P < .001$  vs Veh, ## and ###,  $P < .01$  or  $P < .001$  vs Veh + CXCL1

### 3.16 | Plasma exposure in CIA model

Orally dosed HM5023507 showed dose-related increases in plasma exposure in animals with arthritic inflammation (Figure 6A, Panel B). The exposures (ng/mL\* $h$ ; Mean  $\pm$  SEM,  $n = 3$ ) in the BID regimen were:  $684 \pm 78$  (0.1 mg/kg),  $1881 \pm 112$  (0.3 mg/kg),  $6562 \pm 600$  (1 mg/kg), and  $20\,928 \pm 1037$  (3 mg/kg), respectively.

The corresponding plasma exposures under the QD regimens were  $1960 \pm 122$  (0.6 mg/kg) and  $19\,617 \pm 1385$  (6 mg/kg), respectively. These results indicate that exposure was related to total dose administered daily, and independent of dosing regimen. For example, plasma exposure seen with 0.6 mg/kg or 6 mg/kg given once day was fairly similar to that seen with 0.3 mg/kg or 3 mg/kg, given twice-a-day.



**FIGURE 5** HM5023507 (p.o.) attenuates concanavalin-A (Con-A)-induced inflammatory cytokine response in female Wistar rats. Rats were given HM5023507 or vehicle (t = 0 h), injected intravenously with Con-A (30 mg/kg) at t = 0.5 h, and euthanized for blood collection at t = 2 h. Levels of serum IFN $\gamma$  were determined using commercial kits, and plasma levels of HM5023507 were measured by LC-MS methods. Data were expressed as mean  $\pm$  SEM (n = 4). Data were analyzed via one-way ANOVA, followed by Dunnett's test (\*\*P < .001 vs Veh; ##P < .01 and ###P < .001 vs Veh + Con-A). In a separate experiment, HM5023507 (10 mg/kg, po; t = 0) attenuated Con-A (30 mg/kg, iv, t = 6.5 h) induced IFN $\gamma$  levels measured at 8 h (ng/mL; mean  $\pm$  SE, n = 4 rats; Naïve: 10  $\pm$  0, Con-A: 394  $\pm$  36, HM5023507  $\pm$  Con-A: 46  $\pm$  4, % Inhibition: 91%; plasma levels of HM5023507 (ng/mL; mean  $\pm$  SD, n = 4) were 2740  $\pm$  612

### 3.17 | Histopathology and micro-CT

Histopathological analyses of hind paws revealed arthritis-induced bone resorption, cartilage damage (structure and cellular infiltration), inflammatory cellular infiltration into peri-articular tissue, synovial inflammation, and hyperplasia (Figure S3). The attenuation of paw edema by HM5023507 was accompanied by changes in histopathological endpoints. In addition, the histopathological improvements were independently corroborated by using micro-CT imaging (Figure 6, Panels C/D). These imaging studies demonstrate attenuation inflammation-induced loss of bone density and increase in surface roughness.

### 3.18 | Collagen-specific Antibody Response

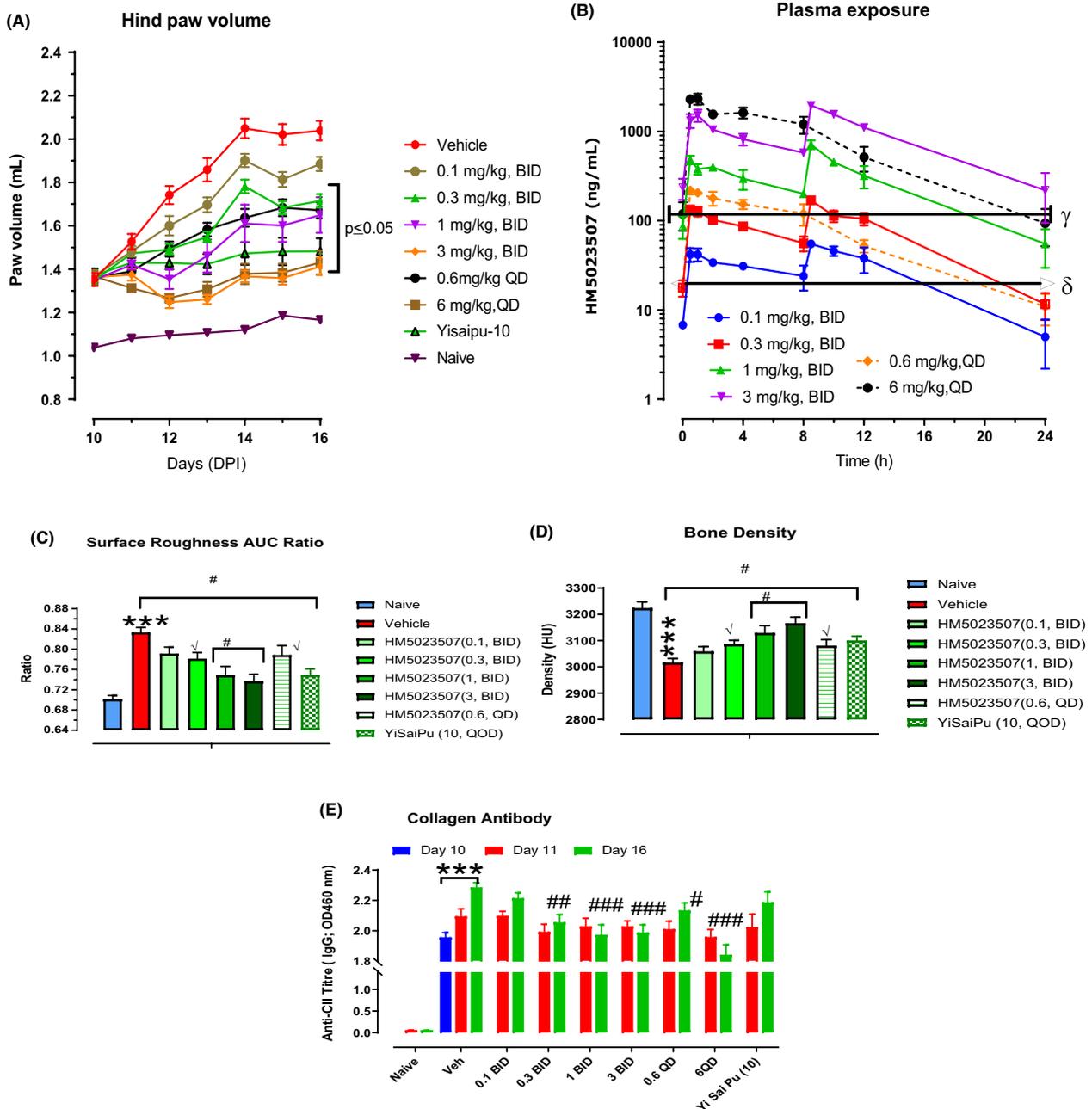
The effect of HM5023507 on the collagen-specific humoral response (ie, anti-collagen antibody production) was examined in serum samples collected at the beginning and the end of the dosing regimen. Arthritic animals showed robust anti-collagen humoral response (Figure 6, Panel E). Treatment with HM5023507 (0.3, 1, and 3 mg/kg BID, and 6 mg/kg QD) significantly attenuated anti-CII IgG levels in the serum as compared to the vehicle group. Under the conditions of the experiment, YiSaiPu® did not affect anti-collagen antibody levels. Since the generation of anti-collagen antibody response requires a productive T: B interaction in the germinal centers, the attenuation of this response by HM5023507 reflects modulation of T: B cross-talk *in vivo*, and further extend the observations seen *in vitro* experiment (Figure 2). Given that HM5023507 dosing was initiated after the collagen immunization, the decreases in collagen antibody levels reflect dampening of an ongoing inflammation-associated humoral response vs its evolution.

The profile of HM5023507 in the rat CIA model demonstrates its robust anti-inflammatory activity presumably mediated via its impact on various immune cells and pathways. As BID or QD regimens, associated with similar AUCs, led to the similar magnitude of reductions in paw inflammation, the anti-inflammatory activity of HM5023507 in the CIA model appears to be driven, in part, by systemic exposure as measured by the AUC.

The estimates of the duration of target coverage based on plasma exposures in the CIA model are summarized in Table 6. In the BID regimen, at the approximate ED<sub>50</sub> dose of 0.3 mg/kg, the plasma concentrations were above the PI3K $\delta$ / PI3K $\gamma$  IC<sub>50</sub> levels for ~24 hours and ~6 hours, respectively. In the QD regimen, at the approximate ED<sub>50</sub> dose of 0.6 mg/kg, the plasma concentrations were above the PI3K $\delta$ / PI3K $\gamma$  IC<sub>50</sub> levels for ~20 hours and ~8 hours, respectively. At doses of  $\geq$  1 mg/kg (BID or QD), the plasma concentrations are above the PI3K $\delta$ / PI3K $\gamma$  IC<sub>50</sub> values for nearly 24 hours. Given the ~eightfold difference in PI3K $\delta$ / PI3K $\gamma$  potency, the magnitude (ie, fold above IC<sub>50</sub> and IC<sub>90</sub> values) and the duration of coverage of PI3K $\delta$  will be influenced both by the dose, and the dosing regimen.

## 4 | DISCUSSION

The efficacy of PI3K inhibitors in human cancers<sup>5,14-16</sup> is consistent with the evidence linking gain of function mutations in PI3Ks ( $\alpha$ ,  $\beta$  and  $\gamma$ ) as drivers of malignancy.<sup>4,25,26</sup> Unfortunately, the efficacy often requires complete/sustained inhibition of PI3K signaling resulting in dose-limiting toxicities (eg, colitis, hepatitis, pneumonitis, hypertension, hyperglycemia, skin rash, and opportunistic infections), likely driven by the disruption of homeostatic innate/adaptive immune PI3K signaling.<sup>17,40</sup> The genetic deletion of either PI3K  $\delta$  or PI3K $\gamma$  or both isoforms in mice



**FIGURE 6** Therapeutic administration of HM5023507 (p.o.) attenuates arthritic paw inflammation in female Wistar rats immunized with bovine collagen. Panel A- Paw edema, and Panel B- Plasma exposure, Panels C & D, micro-CT analysis of hind paws for changes in bone density (measured by Hounsfield units, HU) or surface roughness area under the curve (AUC), respectively, and Panel E - anti-collagen antibody titers (sera diluted 10,000 x). The hind paw volumes were measured daily by plethysmometer and changes in average paw volumes were depicted over time in rats dosed with vehicle, or HM5023507 (0.1, 0.3, 1.0, 3.0 mg/kg, p.o., BID or 0.6 or 6.0 mg/kg, p.o.) or YiSaiPu® (10 mg/kg, i.p., QOD). Paw volumes were analyzed by repeated measured ANOVA analysis followed by Fisher's Least Significant Difference (LSD) test. Data represent mean  $\pm$  SEM ( $n = 10$ , except naïve where  $n = 4$  animals). All other data were analyzed by ANOVA analysis followed by Dunnett's test (\*\* $P < .001$  vs naïve, #, ##, ### $P < .05$ ,  $P < .01$  and  $P < .001$  vs Veh [Panels C-E] √,  $P < .05$  (t-test vs Veh alone)

or hyperactivation of PI3K $\delta$  in humans<sup>41</sup> leads to exaggerated immune-inflammatory responses at the expense of protective, physiological immunity. Thus, identification of dual  $\delta/\gamma$  inhibitors with desirable drug-like properties and acceptable safety profile to support clinical exploration in chronic autoimmune conditions continues to be a formidable challenge. As such, the

promise of PI3K $\delta/\gamma$  inhibitors (or inhibitors of any PI3K isoform) as a novel autoimmune therapeutic is yet to be realized.

The critical success factors for our dual inhibitor program were: (a) a balanced dual inhibition of PI3K $\delta/\gamma$ , (b) broad selectivity against other targets including kinases, GPCRs and ion-channels, (c) consistent target biology/ engagement across a range of

**TABLE 6** PI3K $\delta/\gamma$  target coverage in the rat CIA Model: Influence of dose and dosing regimen

Dose regimen (mg/kg, p.o.)	PI3K $\delta$ coverage (hr above IC <sub>50</sub> / IC <sub>90</sub> *)	PI3K $\gamma$ coverage (hr above IC <sub>50</sub> / IC <sub>90</sub> *)
0.1 BID	7/0	0/0
0.3 BID	24/x	~6 hr/0
0.6, QD	20/~3	~8/0
1 BID	~24/~16	~24/0
3 QD	24/24	24/~5

Note: PI3K $\delta$  IC<sub>50</sub>/ IC<sub>90</sub>\* (IgD-induced B cell activation assay): 45 nmol/L /450 nmol/L\*; PI3K $\gamma$  IC<sub>50</sub>/ IC<sub>90</sub>\*: 250 nmol/L /2500 nmol/L (CXCL1- $\alpha$  induced air pouch neutrophil chemotaxis assay). \*IC<sub>90</sub> values were approximated as 10x IC<sub>50</sub> (assuming n<sub>H</sub> = 1).

assay systems, with an emphasis on human translation, and (d) robust pharmacodynamic activity in preclinical models at exposures consistent with target engagement to enable human dose predictions.

In *in vitro* assays, HM5023507: (a) is a dual inhibitor of PI3K $\delta$  and PI3K $\gamma$  isoforms with selectivity against other members of class I PI3K (PI3K $\alpha$  and PI3K $\beta$ ) or class II/III isoforms and broad kinome selectivity, minimal off-target activity in ion-channel and GPCR screening, (b) inhibits PI3K $\gamma$  and PI3K $\delta$  isoforms in isolated immune cells and in complex milieu such as the blood, and (c) attenuates T cell and B cell activation. The overall selectivity profile of HM5023507 allowed us to associate its pharmacology as on-target (PI3K $\delta/\gamma$ ) driven.

Consistent with the known PI3K $\delta/\gamma$  biology,<sup>10,11,13,30,38</sup> HM5023507 modulated diverse immune responses such as activation of innate and adaptive immune cells, markers of myeloid cell migration, cytokine secretion, and differentiation of naïve T cells into T-helper cells. HM5023507 was equally potent in blocking the production of Th17 cytokines such as IL-17A, IL-22, and IFN $\gamma$  in blood from healthy subjects and rheumatoid arthritis patients suggesting that its pharmacology was not significantly skewed by the disease setting. The impact of HM5023507 on TH17 biology is noteworthy in light of the pathological role of this pathway in several immune-mediated diseases. The relative lack of effect HM5023507 on Treg function *vis-a-vis* Th1, Th2, and Th17 suggest a potential for it to spare Treg function. The impact of PI3K inhibition on Treg function was found to be highly context dependent with contrasting results reported in the literature.<sup>34,36</sup> Additional studies are needed to clarify the impact of PI3K $\delta/\gamma$  inhibition on Treg function in autoimmunity.

HM5023507 is orally bioavailable and potently attenuated PI3K $\delta$  or PI3K $\gamma$  signaling *in vivo*, as reflected in reductions Ig-D-induced B cell activation, CXCL-1-induced neutrophil migration into air pouch<sup>13</sup> and Con-A-induced serum IFN $\gamma$  responses<sup>29</sup> in the rat. The rank order of potency of inhibition of B cell activation and neutrophil migration by HM5023507 (ED<sub>50</sub> values of ~ 0.14 mg/kg, PO and 3 mg/kg, PO, respectively) *in vivo* in rat mirrors the PI3K $\delta/\gamma$  inhibitory ratio of 1:8 in human basophil assay, *in vitro*.

The robust anti-inflammatory activity of HM5023507 in the CIA model is consistent with the role of PI3K $\delta/\gamma$  isoforms in autoimmune

pathways.<sup>7,13,42-44</sup> Interestingly, QD and BID dosing regimens that resulted in similar plasma exposures, but differing degrees of PI3K $\delta/\gamma$  coverage (Table 6) provided similar inhibition of paw inflammation. The reductions in collagen antibody in the CIA model are consistent with the role of PI3K $\delta$  (>PI3K $\gamma$ ) on B cell function and/or T: B cross talk,<sup>20,30</sup> and with its effects on IgG production in T/B cocultures *in vitro* (BioMap™ assay). The attenuation of IgG production by seletalisib, a PI3K $\delta$  selective inhibitor, in BioMAP™ T:B cocultures<sup>10</sup> further supports the role of PI3K $\delta$  in T:B cross talk.

Discovery of PI3K $\gamma$  specific inhibitors or dual  $\delta/\gamma$  inhibitors has faced the challenge of isoform selectivity due to the high homology between PI3K $\gamma$  and PI3K $\alpha$ . The precise PI3K $\delta/\gamma$  inhibitory ratio for a safe and effective autoimmune therapeutic is unknown; however, we targeted an idealized potency ratio (~1:1). This campaign was driven by medicinal chemistry efforts enabled by X-ray crystallography and computational modeling, a battery of optimized biochemical/cellular/whole blood assays, and finally pharmacodynamic/mechanistic models suited to interrogate the target biology *in vivo*.<sup>28</sup> With over 1000 compounds synthesized, profiled and optimized for drug-like properties, identification of balanced dual inhibitors remained a formidable challenge. HM5023507, the most advanced compound, showed the desired 1:1 inhibitory potency against PI3K $\delta/\gamma$  isoforms in *in vitro* kinase assays. However, a shift in PI3K $\delta/\gamma$  inhibitory potency was observed in cellular and whole blood assays. Based on human basophil activation assay, HM5023507 is characterized to be a dual PI3K $\delta/\gamma$  inhibitor with a selectivity ratio of ~1:8. The *in vivo* studies highlighted the influence of dose, dosing regimen and pharmacokinetics of HM5023707 on the magnitude and duration of PI3K isoform inhibition, therefore, target coverage/selectivity. The study highlights the importance of integration of *in vitro* and *in vivo* results, and pharmacokinetics for a holistic definition of isoform selectivity.

In summary, HM5023507 represents a highly selective, dual PI3K $\delta/\gamma$  inhibitor with drug-like properties and robust *in vitro/ in vivo* pharmacology, coupled with consistent, translatable biology. This overall profile makes it a useful tool to study the biology of PI3K  $\delta/\gamma$  signaling.

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## CONFLICT OF INTEREST

The work was conducted under a research collaboration between Hutchison MediPharma or Janssen Pharmaceuticals R&D, LLC., and the authors are employees of respective organizations.

## AUTHOR CONTRIBUTIONS

YC, GD, XL, YS, WPL, JV, JPE, WS, and TR participated in study design. YC, JY, PR, JH, HW, KX, HJ, JW, KN, GC, and PDA conducted experiments. GD, WS, JV, and JPE contributed to reagents. YC, WPL,

TR, and PDA performed data analysis. WPL, PDA, and TR wrote or contributed to the writing of the manuscript. All authors have access to the data/results and reviewed the manuscript.

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### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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