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Structure–activity relationship study, target identification, and pharmacological characterization of a small molecular IL-12/23 inhibitor, APY0201

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ABSTRACT

Interleukin-12 (IL-12) and IL-23 are proinflammatory cytokines and therapeutic targets for inflammatory and autoimmune diseases, including inflammatory bowel diseases, psoriasis, rheumatoid arthritis, and multiple sclerosis. We describe the discovery of APY0201, a unique small molecular IL-12/23 production inhibitor, from activated macrophages and monocytes, and demonstrate ameliorated inflammation in an experimental model of colitis. Through a chemical proteomics approach using a highly sensitive direct nanoflow LC–MS/MS system and bait compounds equipped with the FLAG epitope associated regulator of PIKfyve (ArPIKfyve) was detected. Further study identified its associated protein phosphoinositide kinase, FYVE finger-containing (PIKfyve), as the target protein of APY0201, which was characterized as a potent, highly selective, ATP-competitive PIKfyve inhibitor that interrupts the conversion of phosphatidylinositol 3-phosphate (PtdIns3P) to PtdIns(3,5)P_2. These results elucidate the function of PIKfyve kinase in the IL-12/23 production pathway and in IL-12/23-driven inflammatory disease pathologies to provide a compelling rationale for targeting PIKfyve kinase in inflammatory and autoimmune diseases.

1. Introduction

Interleukin-12 (IL-12, also called IL-12p70) is a heterodimeric proinflammatory cytokine comprising two subunits, named p35 and p40, and is involved in type 1 helper T (Th1) cell-mediated inflammation.[1] IL-12 induces interferon-gamma (IFN-γ) production from NK and T cells, which contributes to phagocytic cell activation as well as inflammation and has an important role in defense against infection and intracellular pathogens. IL-23, a more recently discovered member of the IL-12 family, shares the p40 protein subunit with IL-12 and promotes Th1 response, but its functions differ from those of IL-12.[2] Furthermore, IL-23 is important in late developmental functions of Th17 cells, which are essential for immune effector activity and pathogenicity.[3,4]

The predominant physiological producers of IL-12 and IL-23 are macrophages and dendritic cells, followed by neutrophils.[5] Previous studies have reported the involvement of the downstream pathway of phosphoinositide 3-kinase (PI3K) in IL-12 production from dendritic cells and monocytes.[6,7] One downstream substrate of PI3K is a Ser/Thr protein kinase mammalian target of rapamycin (mTOR), which acts as a negative regulator of IL-12 production by production of IL-10. Moreover, glycogen synthase kinase 3 (GSK3),

Abbreviations: ArPIKfyve, associated regulator of PIKfyve; DNLC, direct nanoflow liquid chromatography; GSK3, glycogen synthase kinase 3; IBD, inflammatory bowel disease; IFN-γ, interferon-gamma; IL-12, interleukin-12; LPS, lipopolysaccharide; mTOR, mammalian target of rapamycin; PBMC, peripheral blood mononuclear cells; PI3K, phosphoinositide 3-kinase; PIKfyve, phosphoinositide kinase, FYVE finger containing; PSL, prednisolone; PtdIns3P, phosphatidylinositol 3-phosphate; SAC, *Staphylococcus aureus* Cowan I strain; SAR, structure–activity relationship; TG-PEC, thioglycollate-induced mouse peritoneal exudate cells; TNF-α, tumor necrosis factor-alpha.

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another downstream target of PI3K pathways, positively regulates IL-12 production through the IL-10-independent pathway.

Although the inflammatory effector functions of Th1 and Th17 are crucial for eliminating pathogens, overproduction of proinflammatory cytokines IL-12/23 leads to development of serious pathological conditions in diverse inflammatory diseases, such as Crohn's disease,[3,8] ulcerative colitis,[3] psoriasis,[9,10] and rheumatoid arthritis.[11] The therapeutic potential of IL-12/23 inhibition has been validated in both clinical and preclinical studies. Previous reports on mice deficient for IL-12p40 or IL-12p19 subunits have proven the central role of IL-12/23 in various inflammatory states in animal models related to colitis,[12] multiple sclerosis,[13] arthritis,[14] and psoriasis.[15] Ustekinumab, which is an injectable monoclonal antibody that neutralizes IL-12p40, has been approved for treating moderate to severe plaque psoriasis,[9,15–17] and it has shown effectiveness in clinical trials in patients with Crohn's disease.[18] Thus, an orally available small molecule capable of inhibiting IL-12/23 production is strongly needed as another option for patients.[19–22]

In the course of phenotypic cell-based screening and directed medicinal chemistry campaigns using the production level of IL-12p70 as index, we generated APY0201 (Compound **1**, Fig. 1a), which possesses a pyrazolo[1,5-*a*]pyrimidine ring as its structural core. APY0201 works differently from anti-IL-12/23 antibodies and acts by inhibiting production of these proinflammatory cytokines with characteristic selectivity over other cytokines, including tumor necrosis factor-alpha (TNF-α). Despite its unique and interesting pharmacological profile, the target protein of APY0201 was unknown.

In this article, we describe our chemical proteomics approach that uses a direct nanoflow liquid chromatography (DNLC) system and bait compounds tagged with the FLAG peptide epitope[23,24] to identify phosphoinositide kinase, FYVE finger-containing (PIKfyve) kinase as the APY0201-binding protein.[25] DNLC is a unique flow system equipped with a novel splitless nanoflow gradient elution device that allows the use of a fritless high-resolution electrospray interface column at an extremely slow flow rate (>50 nL/min). A combination of this pressure-resistant (approximately 100 kg/cm^2) LC system with data-dependent collision-induced dissociation tandem mass spectrometry (MS/MS) on a Q-TOF hybrid mass spectrometer plus automated data processing facilitated identification of protein components in low femtomole amounts of a functional multiprotein complex that was prepared from one dish of cultured cells.

PIKfyve is a mammalian type III phosphatidylinositol phosphate (PIP) lipid kinase that acts on PtdIns3P to generate phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P_2). PIKfyve has an important role in pleiotropic cell functions, such as intracellular compartment morphology, membrane trafficking pathways, and endosomal dynamics;[26,27] inhibition of this kinase led to endosomal enlargement and cytoplasmic vacuolation due to impaired PtdIns(3,5)P_2 synthesis.[28,29] However, no report had indicated the immunological function of PIKfyve concerning the regulation of IL-12/23 production and therapeutic potential as an anti-inflammatory drug target until we disclosed this PIKfyve-IL-12/23 pathway in the patent article[30] and conference papers[31,32] in 2011 and 2012. In the present work, we performed pharmacological evaluation of APY0201to determine the effect of selective inhibition of PIKfyve kinase on immunomodulatory activity and anti-inflammatory potential.

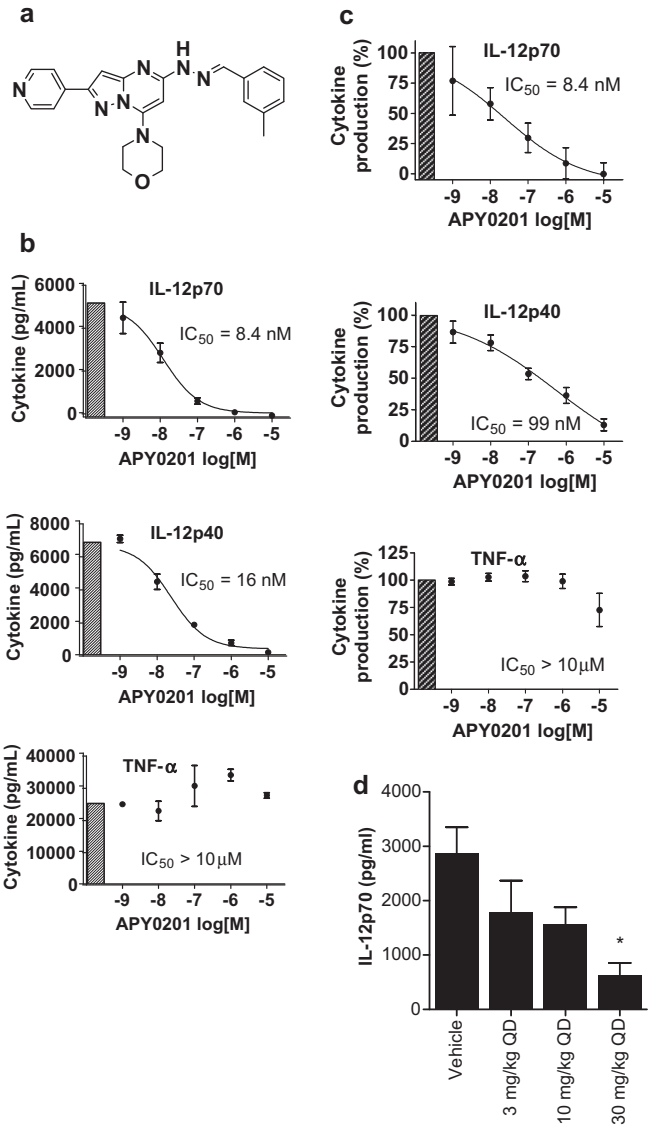


Figure 1. APY0201 selectively inhibits IL-12/23 production. (a) Chemical structure of APY0201. (b) Inhibition profiles of the cytokine production of APY0201 in mouse TG-PEC. The cells were stimulated by 100 ng/mL IFN-γ and 0.05% (v/v) SAC with the indicated concentrations of APY0201. Supernatants were tested for IL-12p70, IL-12p40, and TNF-α. The black columns show activated conditions in the presence of the vehicle control. Data are expressed as means ± sds. in at least three experiments. (c) Human PBMC from healthy volunteers (n = 3) were exposed to either the control or to APY0201 and stimulated as described in (b) to evaluate cytokine production. Black columns show activated conditions in the presence of the vehicle control. Data were normalized to the detected cytokine levels for each volunteer in activated hPBMC (139.2, 201.7, and 592.8 ng/mL for IL-12p70; 419.8, 555.2, and 902.1 ng/mL for IL-12p40; 13,226, 14,731, and 16,281 ng/mL for TNF-α). Data are shown as means ± sds. for three experiments. (d) Shown is the APY0201 inhibition of IFNγ/SAC-stimulated IL-12p70 production in an ex vivo experiment using BALB/c mice (n = 8). ELISA was used to measure the amount of IL-12p70 in the whole blood drawn 30 min after the subjects received oral administration of APY0201. *P < 0.005 compared with the respective vehicle-treated group.

2. Results

2.1. APY0201 showed selective inhibition of IL-12/23 production

IFN-γ priming is considered to be critical for IL-12 production of murine macrophages and human monocytes by costimulation with lipopolysaccharide (LPS) or the *Staphylococcus aureus* Cowan I strain (SAC).[33] Using IFN-γ/SAC as a stimulant (Fig. 1b and c), cell-based assays in thioglycollate-elicited mouse peritoneal macrophages or human peripheral blood mononuclear cells (PBMC) were performed to evaluate the production of IL-12p70, IL-12p40, and TNF-α to identify the inhibitory profile of APY0201 for cytokines. In stimulated thioglycollate-induced mouse peritoneal exudate cells (TG-PEC), APY0201 strongly inhibited IL-12p70

and IL-12p40 production, with IC_{50} values of 8.4 and 16 nM, respectively. Potent inhibition for IL-12p70 production was also observed in human PBMC at the same IC_{50} value as observed in TG-PEC, which implied that the inhibitory activity of APY0201 on IL-12p70 was similar in rodents and humans. APY0201 also inhibited IL-12p40 at 99 nM in human PBMC. APY0201 showed significant selectivity for the production of IL-12p70 and IL-12p40 over TNF-α, and this selectivity was maintained across species. To date, some small molecules, such as phosphodiesterase 4 inhibitors,[34] toll-like receptor 4 signaling inhibitor,[35] and 14-3-3ζ modulators,[19] have shown inhibition of IL-12/23 production, but none of these compounds have a reported mode of action with the characteristic selectivity observed for APY0201.

Mouse whole blood was drawn 30 min after oral administration of APY0201; it was then incubated with a mixture of IFNγ and SAC to induce cytokine production (Fig. 1d) for examining the ex vivo response of IL-12p70 production. Oral APY0201 at a 30 mg/kg dose showed significant reduction of IL-12p70 production (78% inhibition relative to that of the vehicle control), which implied that the inhibitory potential of APY0201 against IL-12 was confirmed in the animal experiment.

2.2. APY0201 bound to the protein complex incorporating the ArPIKFyve

The unique cytokine selectivity profile and oral efficacy of APY0201 led us to investigate the mode of action of this compound. To explore the APY0201 binding protein, we adopted a chemical proteomics approach that included a comprehensive analysis of compound–protein binding by affinity-based isolation of the complex (Fig. 2).

To identify the binding proteins of our IL-12/23 production inhibitors, substitution of a pyridine moiety connected to the pyrazolo[1,5-*a*]pyrimidine ring of APY0201 was investigated to design bait compounds tagged with the FLAG epitope at the appropriate position (Table 1). A simple phenyl group was tolerated as replacement for the pyridine ring (**2**) in terms of inhibition of IL-12p70 production as well as of selectivity over TNF-α. The compound **2** showed 82% inhibition for the expression level of IL-12b mRNA on mTG-PEC cells stimulated with SAC/IFNγ in our preliminary experiment. Further introduction of piperadine on the phenyl group in compound **2** (**3**) showed activity equipotent to that of **2**. Further acylation of the secondary nitrogen atom in the piperidine ring in **3** (**4** and **5**) showed that the activity and selectivity of APY0201 was maintained, which indicated the possibility of tethering at this position through the acylpiperidine structure. Replacement of the *m*-tosylhydrazone of APY0201 with a simple chlorine atom (**6**) resulted in dramatic loss of inhibition against

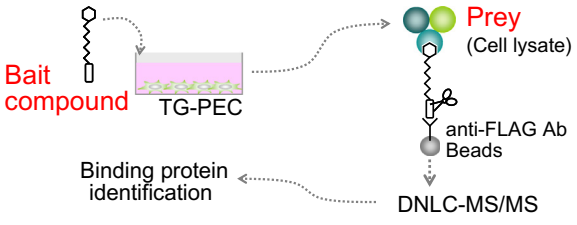


Figure 2. Shown is the schematic strategy for a chemical proteomics approach to target identification using a highly sensitive DNLC–MS/MS system and a bait compound equipped with the FLAG peptide epitope. Cell lysate was prepared from mouse TG-PEC cells incubated with a mixture of IFNγ and SAC as stimulants. Bait compound was added to capture the interacting protein complex. The bait compound–protein complex was purified by immunoaffinity chromatography with the anti-FLAG antibody. Proteins in the column eluate were digested with Lys-C endoproteinase, and DNLC–MS/MS was used to analyze the obtained mixture of peptides to identify the binding proteins of inhibitors of IL-12/23 production.

IL-12p70 production, which implied that this pharmacophore is crucial for the activity.

On the basis of the obtained structure–activity relationships (SAR), **3** and **6** were selected as 'warheads' and incorporated into positive and negative bait compounds, respectively. Two linkers, C_{12}- and PEG-based types that possessed different physicochemical properties (Fig. 3), were used to connect the FLAG epitope. These four bait compounds, **7**–**10**, were added into cell lysate prepared from IFNγ/SAC-stimulated TG-PEC to capture the interacting protein. Affinity purification with an anti-FLAG antibody was used to purify the bait compound–protein complex. Proteins in the eluate were digested with Lys-C endoproteinase, and DNLC–MS/MS was used to directly analyze the resulting peptide fragments to identify bait compound-associated proteins. Sixty-seven proteins were found using this approach (Table 2 and the Supplementary information).

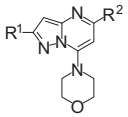
Previously, we experienced that many of these detected proteins were not the true binding protein of the target compound, probably because some proteins interact with bait compounds non-specifically or bind to parts other than the critical 'warhead' moiety. Thus, only the case with 'Found' for both positive control bait compounds (**7** and **9**) was considered to be a 'true positive', and only the case with 'Negative' for both negative controls (**8** and **10**) was considered to be a 'true negative'. Consequently, only the VAC14 homolog, also known as the associated regulator of PIKfyve (ArPIKfyve),[36] was found with both positive bait compounds but not with both negative compounds.

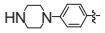
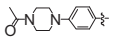
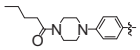
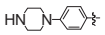
2.3. APY0201 was identified as an ATP-competitive inhibitor of PIKfyve kinase

ArPIKfyve is a scaffold protein that exists as a trimolecular complex with at least two proteins, PIKfyve and Sac domain-containing phosphatase 3 (Sac3), and predominantly localizes in late endosomes and lysosomes of mammalian cells.[29] This homolog physically associates with PIKfyve and Sac3 on the basis of co-immunoprecipitation assays with endogenous or overexpressed proteins;[27] thus, the obtained result could be considered to mean that APY0201 binds to the protein complex incorporating the ArPIKfyve. This protein complex has a significant role in the biosynthesis and turnover of the signaling lipid PtdIns(3,5)P_2, which is a low abundance phosphoinositol that was reported to be an essential mediator of critical endocytic membrane homeostasis.[37] PIKfyve is stimulated by a regulatory complex containing ArPIKfyve and Sac3, whereas PtdIns(3,5)P_2 degradation is catalyzed, at least in part, by the phosphatase activity of Sac3.

In 2008, Jefferies reported the PIKfyve inhibitor YM201636 (Fig. 4a) that blocked PtdIns(3,5)P_2 production and reversibly impaired endosomal trafficking in NIH3T3 cells, which mimicked the effect induced by depleting PIKfyve with short interfering RNA (siRNA).[38] This compound was found through an investigation to determine ATP-competitive PI3K p110α inhibitors possessing critical morpholine in their structures.[39] The morpholine ring is a frequently used substructure of several PI3K inhibitors that allows formation of a hydrogen bond between a morpholino oxygen atom and an NH proton of the 'hinge' region in the ATP-binding site of the targeted kinase.[40] YM201636 has a morpholino-pyrimidine scaffold that is common to APY0201. These two findings, that is, the possibility of a PIKfyve–ArPIKfyve–Sac3 complex acting as a binding protein of APY0201 and the structural overlap between YM201636 and APY0201, suggested the hypothesis that APY0201 would block the function of PIKfyve and PIKfyve is the key regulating enzyme for IL-12/23 production. The inhibitory activity of YM201636 for the production of IL-12p70 in IFNγ/SAC-stimulated TG-PEC was tested and showed negligible influence on TNF-α production up to 10 μM (details are provided in the Supplementary

Table 1
Structure–activity relationship (SAR) study on APY0201 (**1**)



Compound	R^1	R^2	IL-12p70 (IC_{50} nM)	TNF-α (IC_{50} nM)
APY0201 (**1**)			8.4 ± 2.5	>10 μM
2			23 ± 0.81	>10 μM
3			21 ± 8.0	>10 μM
4			4.8 ± 2.7	>10 μM
5			18 ± 0.68	>10 μM
6			>2000	>10 μM

Modifications were made at the *para* position of the pyridine ring in APY0201 to identify the appropriate position for tethering the FLAG peptide epitope. Compound **3** was introduced into bait compounds as the positive control, and **6** was used as the negative control.

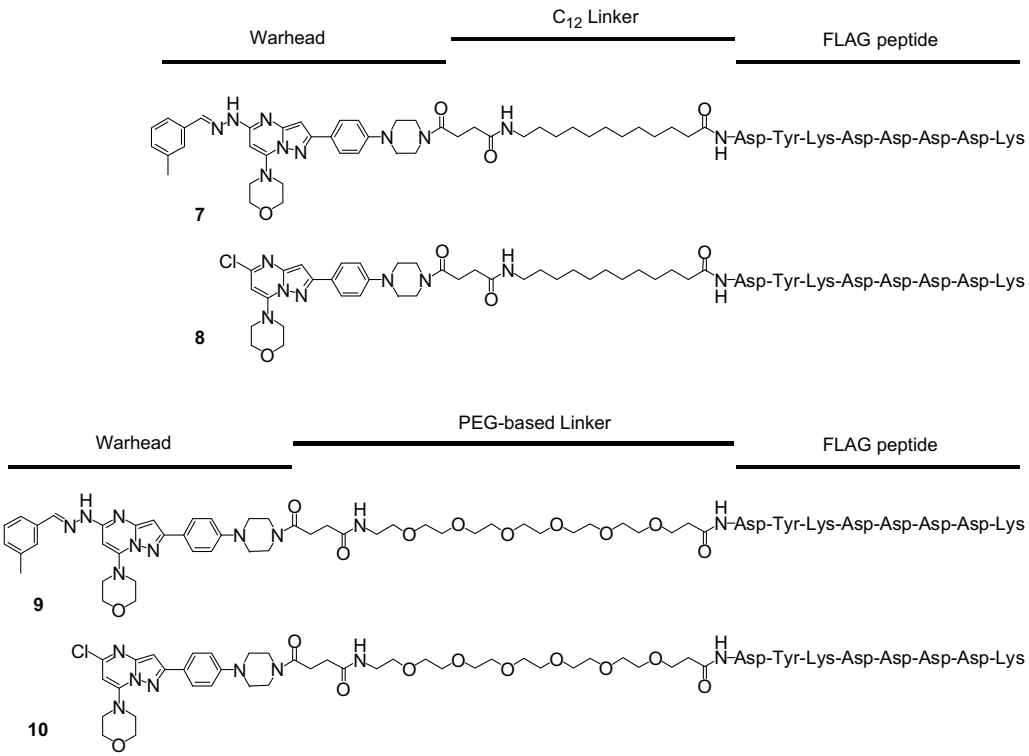


Figure 3. Shown are the bait compounds for target identification. The compounds were designed to have a 'warhead' moiety and the FLAG peptide epitope connected with two different types of linkers. A lipophilic C_{12} linker was used for compounds **7** and **8**, and a hydrophilic PEG-based linker was introduced in compounds **9** and **10**. Bait compounds **7** and **9** were used as positive controls, and **8** and **10** were designed as negative controls.

information). This inhibitory profile for cytokine production was similar to the one for APY0201.

Thus, we next used in situ native kinase profiling with ATP-competitive biotinylated ATP acyl-phosphate probes for 24 lipid kinases and 83 protein kinases to evaluate the binding affinities of APY0201 in the ATP binding pocket of a panel of kinases, including PIKfyve, in a lysate of Jurkat cells (Fig. 4c, more details are provided in the Supplementary information).[41,42] This assay platform is a powerful tool for studying the behavior of any lipid and protein kinase in a nearly intact cellular context with endogenous binding partners and post-translational modifications. The system using cellular lysate was suitable for evaluation of the interaction between compounds and PIKfyve, which works as a fully functional kinase only in the trimolecular complex with ArPIKfyve and Sac3.[29] Native kinase profiling revealed that APY0201 inhibited 96.1% and 99.6% of PIKfyve–probe binding at 30 and 300 nM, respectively, and other than PIKfyve, only the kinases ITPK1 and LOK consistently displayed >50% inhibition when treated with

Table 2
FLAG-tagged bait compounds **7**–**10** and DNLC–MS/MS analysis were used to identify proteins in immunoprecipitates prepared with the anti-FLAG from activated mouse TG-PEC cells

Gene symbol	Protein name	Bait compound				Score
		C_{12} Linker		PEG Linker		
		7	**8**	**9**	**10**	
VAC14	ArPIKFyve/VAC14	Found	—	Found	—	4
JAK1	Janus kinase 1	Found	Found	Found	—	3
ANXA2|ANXA2P2	Annexin A2 or pseudogene 2	Found	—	—	—	3
ACTA2|ACTB|ACTBL2|ACTC1|ACTG1|ACTG2	Alpha 2 actin or beta actin or actin beta-like 2 or cardiac muscle alpha actin 1 or actin gamma 1 propeptide or actin gamma 2	Found	—	—	—	3
ARPC5	Actin-related protein 2/3 complex subunit 5	Found	—	—	—	3
FECH	Ferrochelatase	Found	—	—	—	3
H3F3A|H3F3B|HIST1H3B|HIST1H3D|HIST1H3F |HIST1H3I|HIST2H3D|HIST2H3PS2|HIST3H3|LOC347376|LOC440093	Histone family	Found	—	—	—	3
MTHFD1L	Methylenetetrahydrofolate dehydrogenase ($NADP^+$ dependent) 1-like	Found	—	—	—	3
MYO1F	Myosin IF	Found	—	—	—	3
NQO2	NAD(P)H dehydrogenase, quinone 2	—	—	Found	—	3
CPT1A	Carnitine palmitoyltransferase 1A liver	—	—	Found	—	3
PHB	Prohibitin	—	—	Found	—	3
SGPL1	Sphingosine-1-phosphate lyase 1	—	—	Found	—	3
VDAC1	Voltage-dependent anion channel 1	—	—	Found	—	3
VDAC1|VDAC3|VDAC4P	Voltage-dependent anion channel 1 or 3 or 4 pseudogene	—	—	Found	—	3
VDAC2	Voltage-dependent anion channel 2	—	—	Found	—	3
VDAC3	Voltage-dependent anion channel 3	—	—	Found	—	3

The analysis was repeated four times, and the observed peaks for at least two different analyses were denoted as 'Found'. The symbol '—' indicates that the corresponding peaks were not observed or observed only once in the four repeated analyses. One point was added in case of 'Found' for positive controls, and '—' was recorded for negative controls; the cumulative points for each protein are shown in the 'Score' column. Only proteins with more than three points are shown. The full list of identified proteins is provided in the Supplementary information.

300 nM of APY0201. No significant inhibitions of APY0201 were observed for PI3Ks, mitogen-activated protein kinases, GSK3β, or mTOR, all of which were reported to have a role in LPS-induced IL-12 production from dendritic cells or monocytes.[6,7] APY0201 was remarkably selective for several other members of the lipid kinases, including the PI4K and PI5K families. The calculated Gini coefficients were 0.860 and 0.753 for 30 and 300 nM APY0201, respectively.[43] This native kinase profiling assay was also performed for compound **3** to prove its selective and ATP-competitive PIKfyve inhibition as well (83.9% inhibition at 300 nM), whereas compound **6** showed negligible inhibition of the binding between PIKfyve and biotinylated ATP acyl-phosphate probe (6.8% inhibition at 300 nM). Furthermore, APY0201 was submitted to Cerep (Seattle, WA) for evaluation by a receptor binding assay at 10 μM, with a panel of 137 G protein-coupled receptors (GPCRs), enzymes, ion channels, and transporters, and it showed negligible inhibitions relative to the IC_{50} value in the PIKfyve kinase assay. These results clearly suggested that APY0201 is a potent, highly selective, ATP-competitive PIKfyve kinase inhibitor that interrupts phosphorylation of PtdIns3P to yield PtdIns(3,5)P_2. As reported,[44] Apilimod (Fig. 4d) is a selective PIKfyve kinase inhibitor, but our data showed its inhibitory activity on some GPCRs (>85% inhibition at A3, alpha2C, M3, KOP, 5-HT1A, 5-HT1B, 5-HT2D, and 5-HT2B in Diversity profile assays in Cerep, Table S5 in the Supplementary information). Due to the unique pyrazolo[1,5-*a*] pyrimidine structure, APY0201 had superior selectivity over Apilimod for these GPCRs, leading to favorable safety profile (Tables S3–S5 in the Supplementary information).

Consistent with these results, the morpholine moiety in APY0201 has a crucial interaction with the hinge region of the kinase domain through a hydrogen bond between the morpholino oxygen and Leu1940 in the PIKfyve homology model (Fig. 5a and b).

2.4. PIKfyve controls biosynthesis of IL-12 p40 by regulating mRNA expression

On the basis of the experimental results described above, we hypothesized that depletion of PIKfyve kinase activity would lead to a decrease in the expression of IL-12p40, which is an integral component unit of both IL-12 and IL-23.[30–32] After we disclosed the preliminary experimental data using PIKfyve-siRNA in the patent article[30] and conference papers[31,32] in 2011 and 2012, the researchers at Novartis confirmed that blocking the phosphotransferase activity of PIKfyve leads to selective inhibition of IL-12/IL-23p40 by regulating mRNA expression in 2013.[44] The researchers in Astellas followed our results.[45]

On the basis of the aforementioned promising profile, APY0201 was further characterized through pharmacokinetic (PK) studies conducted in mice. The PK parameters obtained after intravenous administration at 3 or 10 mg/kg and oral dosing at 30 mg/kg are shown in Figure 6. For intravenous administration, the PK parameters of initial drug concentration (C_0) and area under the curve to time infinity (AUC_{inf}) showed good linearity between the two doses, and APY0201 showed low total body clearance (CL_{tot} = 1.0 L/h/kg) (Fig. 6a). After oral administration, heart and portal plasma concentrations were determined to assess the first-pass effect (Fig. 6b). The maximum APY0201 concentration (C_{max}) value in the heart was 7.2 μM, and the plasma concentration was 3.5 μM at 6 h. The apparent fraction absorbed and availability at the liver were estimated to be 68% and 76% of the dose, respectively, which indicated a good bioavailability of 52% (Table 3). Because good bioavailability with oral administration was obtained, APY0201 was examined for its oral therapeutic efficacy for once-daily dosing at 30 mg/kg.

2.5. APY0201 ameliorates inflammation in an experimental model of colitis

To determine the impact of PIFKfyve inhibition on inflammatory responses in vivo, we evaluated APY0201 in a mouse model of inflammatory bowel disease (IBD) induced by the adoptive transfer of IL-10 knockout (KO) $CD4^+$ T cells.[46,47] In this model, splenocytes and mesenteric lymph node cells were collected from diseased IL-10 deficient mice, and $CD4^+$ T cells were purified up to

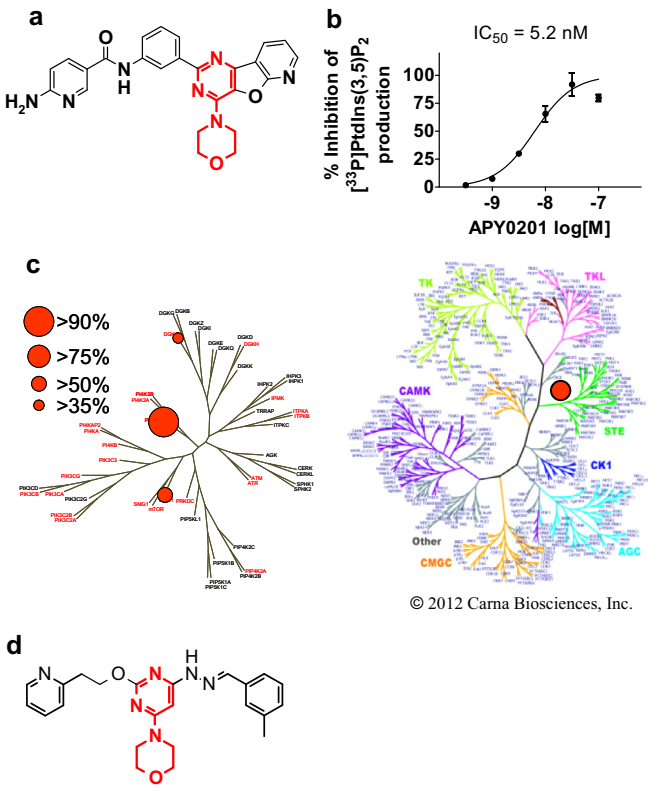


Figure 4. (a) Chemical structure of YM201636. The key morpholino-pyrimidine scaffold is highlighted in red. (b) Cell-free functional assay of full-length PIKfyve kinase. APY0201 inhibited the conversion of PtdIns3P to [^{33}P]PtdIns(3,5)P_2 in the presence of [^{33}P]ATP. Data are presented as mean ± sd from triplicate experiments. (c) Lipid (left) and protein (right) kinase selectivity profile for APY0201 shown on the human lipid and protein kinome dendrogram. An in situ native kinase profiling approach (KiNativ profile) using an ATP-competitive probe and Jurkat cells lysate was used to determine the selectivity for 24 lipid kinases and 83 protein kinases. The lipid kinases evaluated for APY0201 in KiNativ profiling are highlighted in red, and the circles represent the percent inhibitions of APY0201 at 300 nM. The lipid kinase dendrogram was generated on the basis of the alignment of the full-length kinases and represented in radial view. The protein kinase dendrogram was adapted with permission from Carna Biosciences, Inc. (d) chemical structure of Apilimod.

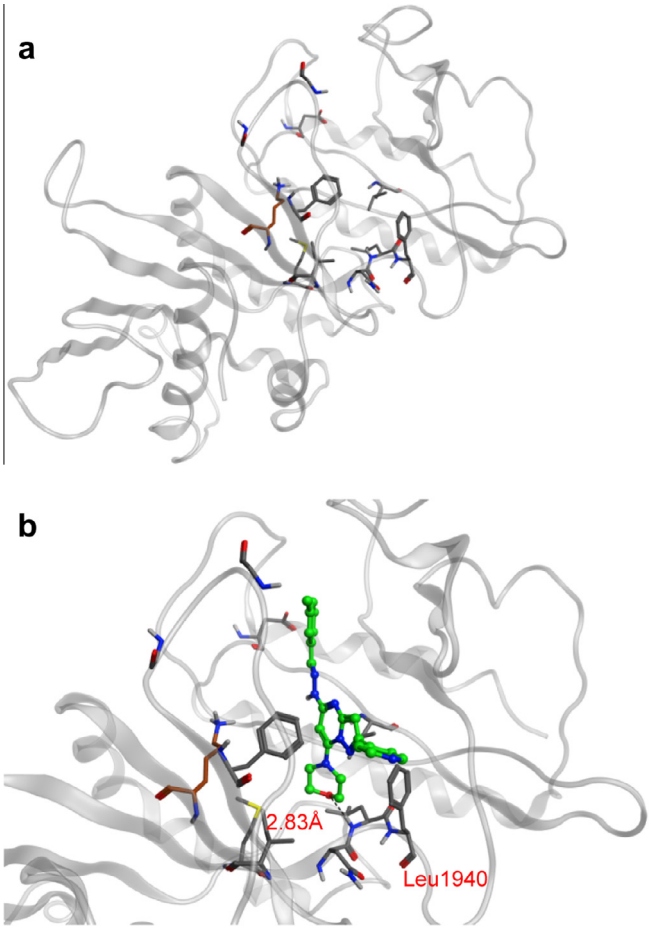


Figure 5. (a) Pictorial representation of the PIKfyve PIPK domain homology model. The amino acid residues in the ATP binding site are explicitly shown. The conserved lysine reacted with an ATP-acylphosphate probe in the KiNativ profiling assay is highlighted in brown. (b) The complex with APY0201 (green) in the ATP-binding site. Critical contact was observed in the morpholino oxygen of APY0201 with Leu1940 (2.83 Å) in the 'hinge' region of the kinase.

95%, which were transferred into female SCID mice to induce spontaneous colitis within 3 weeks. The severity of colitis was assessed by wet colon weight. Stool consistency was scored to evaluate the therapeutic effect of APY0201 on colitis. The animals were sacrificed on day 21, and colon weights were measured. An increase in the colon weight ratio was observed in the vehicle control group for colitis compared with that in the normal control group. Daily administration of APY0201 significantly reduced increases in colon weight in a dose-dependent manner (Fig. 7a; 19.7%, 25.4%, and 73.3% reduction at 3, 10, and 30 mg/kg, respectively), and the effect of APY0201 at 30 mg/kg was equivalent to that of prednisolone (PSL) at 15 mg/kg B.I.D. (81.0% reduction, Fig. 7b). Examination of the stool consistency showed that the vehicle control group exhibited severe diarrhea on the day of the sacrifice, while APY0201 significantly prevented development of diarrhea in a dose-dependent manner (Fig. 7c and d). Twice a day administration of PSL ameliorated inflammation in this mouse model of colitis. However, treatment with PSL led to a significant decrease in body weight after chronic administration relative to that of the vehicle or normal control groups, although the drug relieved diarrhea and reduced colon weight (please see the Supplementary information). Conversely, administration of APY0201 caused no difference in body weight relative to that of the normal control group. These results clearly demonstrated that APY0201 was orally available and significantly efficacious in a mouse model of colitis induced by adoptive transfer of IL-10 KO CD4$^+$ T cells without showing any sign of adverse effects.

3. Discussion

We identified the novel anti-inflammatory drug target PIKfyve kinase that exists as a complex with ArPIKfyve and Sac3 and localizes primarily in late endosomes/lysosomes. In our chemical proteomics approach using compound **3** as a 'warhead' in a positive control bait compound, only ArPIKfyve was found to be a true positive, and direct interaction of compound **3** with PIKfyve was not observed (Table 2). However, the in situ native kinase profiling assay using an ATP acyl-phosphate probe demonstrated APY0201 to be a selective, ATP-competitive PIKfyve kinase inhibitor, and the cell-free kinase functional assay using full-length PIKfyve enzymes showed that APY0201 extensively reduced the kinase activity of the PIKfyve enzyme. These data suggested that APY0201 directly interacted with PIKfyve kinase at the ATP-binding site, which led to suppression of the conversion of PtdIns3P to PtdIns(3,5)P_2 to suppress the production of IL-12/23. Therefore, the results implied that our target binding approach captured the trimolecular protein complex of PIKfyve–ArPIKfyve–Sac3; however, only ArPIKfyve was detected among the three proteins, probably because of the sensitivity limit of the DNLC–MS/MS system or

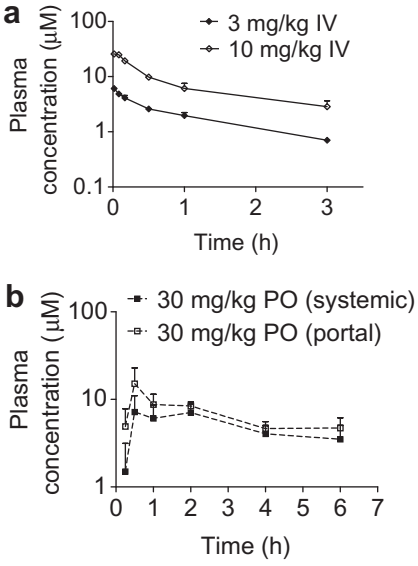


Figure 6. Plasma concentration (mean ± sd) after (a) intravenous and (b) oral administration of APY0201. Female BALB/c mice ($n = 3$) were dosed intravenously at 10 mg/5 mL/kg in 50% PEG 400/50% water titrated with 1 N HCl at 3 or 10 mg/kg and orally as a crystalline suspension in 0.5% methyl cellulose at 30 mg/10 mL/kg. After oral administration, the heart and portal plasma concentrations were separately determined to assess the apparent fraction absorbed.

Table 3
(c) Pharmacokinetic, blood-to-plasma concentration ratio (Rb), and plasma protein binding (PPB) data for APY0201

Intravenous dose (mg/kg)	3	10
C_0 (μM)	6.2	27.6
V_1 (L/kg)	1.2	0.9
AUC_{inf} (μg h/mL)	2.9	12.2
CL_{tot} (L/h/kg)	1.0	0.8
Rb	1.1	
PPB (%)	92	
Oral		
Dose (mg/kg)	30	30
	Heart	Portal
C_{max} (μM)	7.2	16.3
AUC_{lim} (μg h/mL)	12.3	16.8
$F_{a,app}$ (%)	68	
F_h (%)	76	
BA,lim (%)	52	

Rb and PPB were measured at 25 μM.

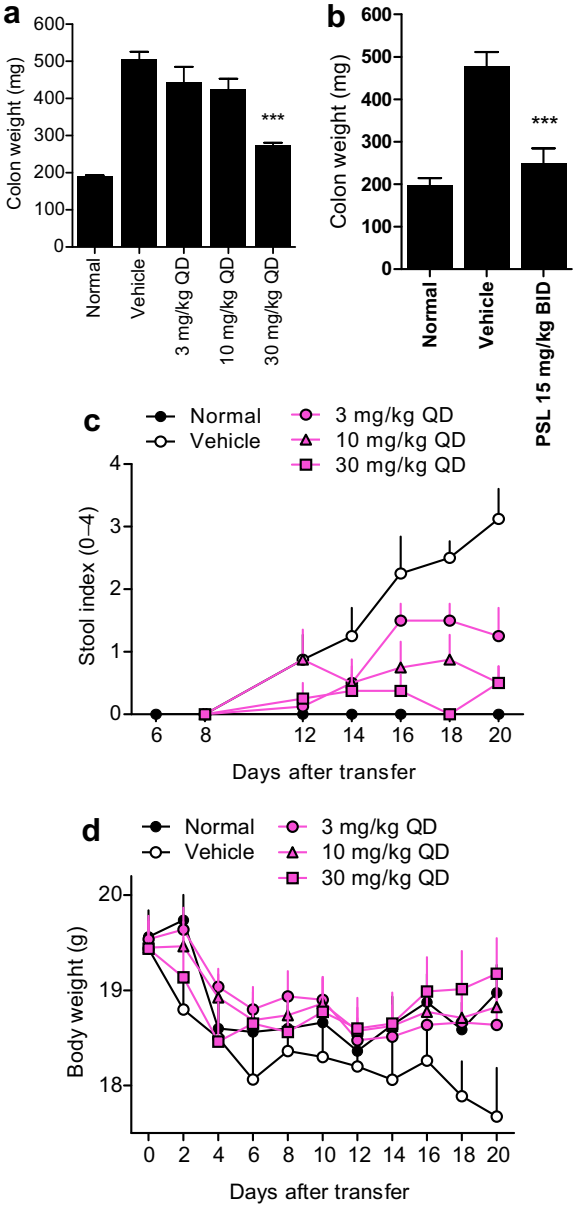


Figure 7. APY0201, a selective inhibitor of PIKfyve and inhibitor of IL-12/23 production, ameliorates colon inflammation in an IL-$10^{-/-}$ cell transfer colitis model. APY0201 or PSL was administered orally to SCID mice with experimental colitis induced by spleen/mesenteric lymph node cells from diseased IL-$10^{-/-}$ mice ($n = 8$). Data are presented as means ± s.ds. (a) APY0201 was administered at 3, 10, and 30 mg/kg daily, and colon weight was measured at 21 days. $^{***}P < 0.0001$ Dunnett's test (vs vehicle). (b) 15 mg/kg PSL was administered twice a day, and colon weight was measured at 21 days. $^{***}P < 0.0001$ Dunnett's test (vs vehicle). (c) Stool consistency was measured at days 12, 14, 16, 18, and 20. The vehicle control group exhibited diarrhea. APY0201 administration prevented diarrhea in a dose-dependent manner. (d) Body weight was measured once every 2 days, and no difference was observed in body weight after APY00201-treatment relative to that in the normal control group.

the amount of protein in the lysate. In the PIKfyve homology model, APY0201 interacts with the 'hinge' region of the ATP-binding site in the kinase domain with its pyridine ring directed toward the outer solvent space (Fig. 5a and b). Thus, tethering the FLAG epitope at the 4-position of the pyridine ring was an appropriate approach for designing bait compounds that may interact with the ATP-binding site of PIKfyve kinase.

The relationship between PIKfyve inhibition and IL-12/23 production inhibition was investigated. Vacuolation is a cellular event strongly associated with decreased levels of PtdIns(3,5)P_2,[38] which was produced from PtdIns3P only as a result of PIKfyve kinase activity. Thus, vacuolation could be a functional biomarker of cellular PIKfyve inhibition and of the reduction of PtdIns(3,5)P_2 in the cell. The EC_{50} values of cellular vacuolation after addition of compounds were consistent with their IC_{50} values for IL-12p70 production, which suggested that these two phenotypic cellular events were simultaneously induced by exposure to a PIKfyve inhibitor. It is reported that depletion of PIKfyve resulted in inhibition of IL-12/23 production at the transcriptional level and provided indications of decreasing cellular PtdIns(3,5)P_2.[44] Therefore, it is strongly suggested that PIKfyve inhibition and cellular PtdIns(3,5)P_2 reduction are closely linked to inhibition of IL-12/23 production from activated macrophages.

Recently, Sbrissa et al. reported that the PIKfyve inhibitor YM201636 reduced not only PtdIns(3,5)P_2, but also PtdIns5P in various cell lines.[48] Our results implied that IL-12/23 production inhibition by APY0201 from activated macrophages is through

PIKfyve inhibition. However, we could not yet elucidate the whole mechanism of observed IL-12/23-inhibitory activity that could include reduction of PtdIns(3,5)P_2, PtdIns3P declination, or other unknown pathways.

Previous reports have stated that a critical phenotype was observed in mice with a null mutation in ArPIKfyve or PIKfyve. The ArPIKfyve-deficient mice showed early postnatal lethality, had 50% of the wild-type PtdIns(3,5)P_2 and PtdIns5P levels, and exhibited massive neurodegeneration with vacuolated cells in multiple regions of the brain.[49] Nearly all PIKfyve homozygous KO mutant mice died at an early stage of embryonic development, with significant reduction of cellular PtdIns(3,5)P_2;[50] however, mice with haploid PIKfyve gene deletion developed normally, with no defects in gross abnormalities, growth rate, and size, although they showed only approximately 60–65% of the wild-type lipid levels.[50] Thus, it is noteworthy that administration of APY0201 at the efficacious dose did not induce signs of adverse effects, and no swollen cells were observed in mice treated with 30 mg/kg that were sacrificed 1 day after the final administration (data not shown). These results suggested that a small molecular PIKfyve inhibitor reversed the kinase activity because it was eliminated from the tested animals. Although a detailed elucidation of the mechanism underlying modulation of IL-12/23 production by PIKfyve inhibition is still being sought, the observed therapeutic margin for APY0201 showed promise for clinical development of the compound as a PIKfyve-selective small molecular inhibitor in IL-12/23–related inflammatory and autoimmune diseases, such as IBD.

APY0201 is an orally-available PIKfyve kinase inhibitor that is selective for all tested kinases, GPCRs, ion channels, and enzymes, and it is a powerful tool for understanding the role of PIKfyve and PtdIns(3,5)P_2 in immunological and inflammatory responses. The administration of APY0201 can be used to assess PIKfyve function in immune cells and animals with normal architecture of the PIKfyve–ArPIKfyve–Sac3 complex. Exposure to APY0201 in vitro blocked production of IL-12/23, which highlighted the effect of selective inhibition of this kinase. The data presented here indicate a unique role of PIKfyve kinase in cytokine production. Because PIKfyve inhibition blocks production of IL-12 and IL-23 from activated macrophages, APY0201 may control the cytokine modulation function of these cell types and reduce the pathological proinflammatory cytokines IL-12/23 with negligible influence on other cytokines, including TNF-α. Moreover, the therapeutic impact of PIKfyve inhibition in a mouse model of IBD included reduction in inflammation without affecting body weight.

In the in vitro assay using mouse whole blood for IL-12p70-inhibitory activity, APY0201 demonstrated potent inhibitory activity against IL-12p70 production with the IC_{50} value of 880 nM. At the therapeutic dose (30 mg/kg daily), the mean C_{max} was 7.2 μM, which is sufficient to inhibit IL-12p70 production in whole blood almost completely, and the estimated plasma concentration at 12 h is still close to 880 nM, which indicated that an appropriate amount of APY0201 was maintained to inhibit IL-12p70 production for nearly a half day (Fig. 6b). Thus, the concentration of APY0201 at this experimental dose could be consistent with the observed therapeutic effect in the IL-10$^{-/-}$ cell transfer colitis model.

In summary, a chemical proteomics approach using a highly sensitive DNLC–MS/MS system and bait compounds equipped with the FLAG epitope was used in this investigation, and further study demonstrated that the associated protein PIKfyve kinase was the possible target protein of APY0201, which is a selective and potent IL-12/23 production inhibitor. The data by researchers at Novartis supported these discussions.[44] Characterization showed that APY0201 was a potent, highly selective, ATP-competitive PIKfyve kinase inhibitor that strongly inhibited IL-12/23 production in vitro and ameliorated inflammation in an experimental model of colitis. The structure–activity relationship study on APY0201, its pharmacological profile in vitro and in vivo, the strategy for target identification, and the biological characterization of PIKfyve kinase as an anti-inflammatory drug target were presented. The results from this investigation should be useful in drug discovery targeted at novel agents for treating autoimmune and inflammatory diseases. Our findings provide a new understanding of the function of PIKfyve kinase in the IL-12/23 production pathway and IL-12/23–driven inflammatory disease pathologies. In addition, these findings support the development of a selective PIKfyve kinase inhibitor as a therapeutic modality for autoimmune disorders, such as IBD.

4. Experimental procedures

4.1. Chemical synthesis

See the Supplementary information for synthetic procedures and characterization data.

4.2. Cell isolation

Mouse TG-PEC cells were collected from female BALB/c mice (6 weeks) as described in the Supplementary information. Human PBMC were isolated from the peripheral blood of healthy volunteers, as described in the Supplementary information.

4.3. Mouse whole blood ex vivo assay

The vehicle (0.5% methyl cellulose) and tested compound were orally administered to female BALB/c mice (6 weeks). After 30 min, the mice were anesthetized, and blood samples were collected by cardiac puncture. The detailed procedures for the preprocessing and analysis are described in the Supplementary information.

4.4. Cell stimulations

Mouse TG-PEC or human PBMC were incubated with the tested compound in the presence of 100 ng/mL mouse or human IFN-γ and 0.05%w/v SAC, as described in the Supplementary information.

4.5. Cytokine measurements

IL-12p70, IL-12p40, and TNF-α levels were determined by ELISA, as described in the Supplementary information.

4.6. Murine IL-10$^{-/-}$ cell transfer colitis

Colitis was induced in female SCID mice ($n = 8$) by adoptive transfer of spleen and mesenteric lymph node cells from diseased IL-10$^{-/-}$ mice, as described previously.[46,47] The tested compound was administered from day 0 to mice with experimental colitis. The mice were sacrificed for assessing inflammation 21 days after cell transfer. The severity of colitis was assessed according to wet colon weight. Scoring of stool consistency was performed once in 2 days (0: normal beaded stool, 2: soft stool, 4: diarrhea). The detailed experimental procedure is provided in the Supplementary information.

4.7. PK profile

Female BALB/c mice ($n = 3$) were used. For intravenous administration, the tested compound was dissolved in 80% PEG 400/20% water (3 or 10 mg/mL/kg) for a dose of 3 or 10 mg/kg, respectively. For oral administration, the tested compound was suspended in 0.5% methyl cellulose (30 mg/5 mL/kg). Blood samples were collected at designated time points by cardiac puncture

(systemic) or from the portal vein (portal) under anesthesia. The detailed procedures for preprocessing, analysis, and calculation of the PK parameters are described in the Supplementary information.

4.8. IC_{50} determination

GraphPad Prism version 5 (GraphPad Software) was used to determine IC_{50}s.

4.9. Statistical analysis

SAS version 6.12 software (SAS Institute, Cary, NC, USA) and Dunnett's method were used to perform statistical analysis, as outlined in the Supplementary information.

4.10. Target identification

Bait compounds equipped with FLAG peptide epitopes (**7**–**10**) were incubated with the cell lysate prepared from IFNγ/SAC-stimulated mouse TG-PEC. The bait compound–protein complex was immunoprecipitated with a bead-bound anti-FLAG antibody, and the bait compound-associated proteins were then digested by Lys-C endoproteinase. A DNLC–MS/MS system was used to analyze the resulting peptides, as described previously.[23,24] The analysis was repeated four times, and the observed peaks for at least two different analyses were denoted as 'Found' in Table 2 and in Table S2 in the Supplementary information. The detailed procedure of the experiment is found in the Supplementary information.

4.11. PIKfyve homology modeling and docking study

The known crystal structure of PIP4KIIβ (PI4K2B, PDB: 1BO1)[51] was used as a template with the Molecular Operating Environment (MOE) software[52] to produce a homology model of PIKfyve kinase. PIP4KIIβ had the closest sequence identity to PIKfyve kinase. The docking function present in the MOE software package was used to dock APY0201 into the obtained homology model of PIKfyve. The detailed procedure is provided in the Supplementary information.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.03.036.

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