Novel Phosphoinositide 3-Kinase δ,γ Inhibitor: Potent Anti-Inflammatory Effects and Joint Protection in Models of Rheumatoid Arthritis

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ABSTRACT

Phosphoinositide 3-kinases (PI3Ks) are members of a lipid kinase signaling family that phosphorylate the 3-hydroxyl position of the phosphoinositide (PI) inositol ring. They are divided into classes I, II, and III according to structural and functional aspects (Yu et al., 1998). Class IA (PI3Kγ and PI3Kδ) are expressed mainly in hematopoietic cells and regulate partially overlapping cell functions in adaptive and innate immunity. Recent approval of a Janus kinase inhibitor and the clinical efficacy of a spleen tyrosine kinase inhibitor for RA support the feasibility of this approach (Weinblatt et al., 2010; Fleischmann et al., 2012; Kremer et al., 2012).

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The potential benefit of targeting PI3K in RA is supported by several studies assessing the roles of PI3K p110γ and p110δ in murine arthritis models. In a collagen type II (CII)–specific antibody-induced arthritis model and in the human tissue necrosis factor transgenic model, Pik3cg−/− mice have decreased arthritis severity and cartilage damage (Pik3cg encodes p110γ). A PI3Kγ inhibitor also reduces clinical severity of arthritis and neutrophil accumulation in a collagen-induced arthritis (CIA) model (Camp et al., 2005). PI3Kγ-deficient mice or administration of PI3Kγ inhibitor in antigen-induced arthritis.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovitis and joint destruction. Treatment with biologic agents such as tumor necrosis factor (TNF) inhibitors has improved outcomes, but many patients have inadequate responses. Orally bioavailable small molecule inhibitors that target signal transduction have emerged as potential alternatives to expensive biologics. Recent approval of a Janus kinase inhibitor and the clinical efficacy of a spleen tyrosine kinase inhibitor for RA support the feasibility of this approach (Weinblatt et al., 2010; Fleischmann et al., 2012; Kremer et al., 2012).

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ABBREVIATIONS: AA, adjuvant arthritis; CIA, collagen-induced arthritis; CII, collagen type II; FLS, fibroblast-like synoviocyte; IL, interleukin; MMP, matrix metalloproteinase; PI, phosphoinositide; PI3K, phosphoinositide 3-kinase; p110δ PI3K, catalytic subunit δ; Th, T helper cell; TNF, tumor necrosis factor.
also decreases clinical arthritis and macrophage migration (Hayer et al., 2009; Gruen et al., 2010). In the K/BxN serum transfer model, p110δ−/− mice have reduced clinical arthritis, histology, and neutrophil infiltrates. Notably, combined p110δ and p110γ deficiency more effectively reduces disease severity than lack of either individual isoform (Randis et al., 2008).

On the basis of these data and the partially overlapping functions of PI3Kγ and PI3Kδ, we evaluated the effect of a potent PI3Kδ,γ inhibitor, IPI-145 (Winkler et al., 2013), on the development and progression of chronic inflammatory arthritis in rat adjuvant-induced arthritis (AA) and mouse CIA. Two separate models in two species were used to profile the pharmacology to assure that the effects are not species or model specific. In mouse CIA, antibody to CII and activation of complement are dominant and genetic background permitting presentation and adaptive immune response to collagen are critical. In AA, the activation of T cells and neutrophils are major mechanisms; however, that of B cells and complement is much less important. The novel inhibitor demonstrated striking benefit, ameliorating initiation and progression of arthritis, reducing synovial inflammation, and decreasing joint damage. These data support development of a PI3Kδ,γ inhibitor such as IPI-145 in RA.

### Materials and Methods

**PI3Kδ,γ Inhibitor**

IPI-145 is a potent PI3Kδ,γ inhibitor, which was provided by Infinity Pharmaceuticals, Inc. (Cambridge, MA). The K₅₀ values for IPI-145 are 0.023 nM for PI3Kδ, 0.24 nM for PI3Kγ, 1.56 nM for PI3Kα, and 25.9 nM for PI3Kε. The four PI3K isozymes were also evaluated using an enzymatic assay that measured [³²P]ATP hydrolysis, demonstrating IC₅₀ values of 2.5 and 27 nM for PI3Kδ and PI3Kγ, respectively, and 1602 and 85 nM for PI3Kα and PI3Kε, respectively (Winkler et al., 2013). Figure 1 shows the chemical structure of IPI-145.

**Rat AA**

Male Lewis rats (180–200 g body weight; Charles River Laboratories, Wilmington, MA) were immunized with 0.1 ml complete Freund's adjuvant supplemented with heat-killed *Mycobacterium tuberculosis* at the base of the tail on day 0 as previously described (Boyle et al., 2001).

**Mouse CIA**

Male DBA/1 mice (aged 6–8 weeks; Harlan Laboratories, Indianapolis, IN) were injected at the base of the tail with 100 μg of bovine CII (Chondrex Inc, Redmond, WA) in 0.05 M acetic acid mixed with complete Freund's adjuvant as previously described (Han et al., 1998). On day 21, 100 μg of bovine CII in phosphate-buffered saline was injected intraperitoneally. On day 28, 5 μg of lipopolysaccharide was administered intraperitoneally to synchronize the disease. Clinical arthritis scores were measured using a semiquantitative scoring system with a maximum score of 16 per each mouse, as previously described (Luo et al., 2011). On day 20, the mice were divided into four groups: 1) vehicle treatment group (n = 7); 2) 10 mg/kg IPI-145 treatment group (n = 8); 3) IPI-145 50 mg/kg early treatment group (n = 8); and 4) IPI-145 50 mg/kg late treatment group (vehicle until day 28, then IPI-145 beginning on day 30; n = 8). The drug dosages were determined by average body weight on day 0. For the first three groups, the vehicle and two doses of drug were injected intraperitoneally once a day from day 20 to day 42. The last group was injected with vehicle from day 20 to day 28, and then 50 mg/kg IPI-145 was administered from day 30 to day 42.

**Microcomputed Tomography**

Ankle joints of rats were imaged using a SkyScan 1076 micro-CT 40 system (Bruker-MicroCT, Kontich, Belgium) with the assistance of Dr. Robert Sah (University of California, San Diego, CA) as previously described (Hammaker et al., 2012). The images were assessed using Quantitative Real-Time Polymerase Chain Reaction

Tissue RNA was isolated from snap-frozen ankle joints using RNA Stat and the RNeasy Lipid Tissue Mini Kit according to the manufacturer's protocol (Qiagen, Valencia, CA). Using cDNA, mRNA

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Fig. 1. Structure of the PI3Kδ,γ inhibitor, IPI-145.
expression of interleukin (IL)-6 and matrix metalloproteinases (MMPs) was measured by the TaqMan Gene Expression Assay (Applied Biosystems, Carlsbad, CA). The threshold cycle (Ct) values were used to calculate the number of cell equivalents using a standard complementary DNA curve as previously described (Boyle et al., 2003). The data were normalized to hypoxanthine guanine phosphoribosyltransferase expression and the results are expressed as relative expression units.

**Western Blot Analysis**

Ankle joints were snap-frozen and protein was extracted using a modified radioimmunoprecipitation assay buffer as previously described (Hammaker et al., 2007). Whole-tissue lysates (15 μg of total protein) were fractionated by NuPAGE 4%–12% Bis-Tris gel electrophoresis (Invitrogen, Carlsbad, CA) and then transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). Nonspecific binding was blocked with 5% nonfat milk in 0.05% Tween 20/Tris-buffered saline for 1 hour at room temperature followed by overnight incubation at 4°C with antibodies to Akt, phospho-Akt, or β-actin (diluted 1:1000; Cell Signaling Technology, Danvers, MA). Anti-β-actin (1:2000; Sigma-Aldrich, St. Louis, MO) was used as a loading control. After washing, the membranes were incubated with horseradish peroxidase–conjugated anti-rabbit secondary antibody (1:2000) for 2 hours at room temperature. Immunoreactive proteins were detected with an Immun-Star conjugated anti-rabbit secondary antibody (1:2000) for 2 hours at room temperature. Densitometric analysis was performed using Quality One 1-D analysis software (Bio-Rad) and normalized relative to the bands of β-actin.

**Serum Antibody Assays**

On day 42, sera of CIA mice were obtained with cardiac punctures. Serum anti-CII total IgG, IgG1, and IgG2a levels were measured using enzyme-linked immunosorbent assay kits according to the manufacturer’s protocols (Chondrex). IgG concentration was measured using a scintillation counter and a stimulation index was calculated. IL-17 concentration in the culture supernatant after 24 hours. The cells were harvested, and 5 × 10^6 splenocytes were stimulated with 50 μg/ml CII CB11 fragment (Chondrex) as previously described (Mohammed et al., 2003; Luo et al., 2011). Because the experiment was designed to explore how IPI-145 affects the role of PI3K in CIA, we evaluated whether the compound decreased articular MMP expression. MMP-13 mRNA levels, but not MMP-3 levels, were significantly reduced in the IPI-145–treated groups compared with the vehicle-treated group (P < 0.001) (Fig. 3). These data suggest that PI3Kδ,γ could protect joints in part by decreasing MMP-13 gene expression.

**Effect of PI3Kδ,γ Inhibition on Akt Phosphorylation in AA.** We then determined the effect of PI3Kδ,γ inhibition on phosphorylation of its downstream target Akt in AA. Ankles were harvested on day 21 and evaluated by Western blot analysis. As shown in Fig. 4, the p-Akt expression was significantly reduced in the IPI-145–treated groups compared with the vehicle-treated group (P < 0.001) (Fig. 3). These data suggest that PI3Kδ,γ could protect joints in part by decreasing MMP-13 gene expression.

**Statistical Analysis**

Results are expressed as the mean ± S.E.M. Statistical differences were assessed using one-way or two-way repeated-measures analysis of variance with Tukey's multiple comparison test. In some cases, the analysis was followed by Dunnett's multiple comparison test or a t test for comparison of each group. A P value < 0.05 was considered significant.

**Results**

**Adjuvant Arthritis in Rats**

**Effect of PI3Kδ,γ Inhibition on the Onset and Progression of CIA.** After evaluating the role of PI3Kδ,γ in AA, we examined their contribution to a model dependent on autotubodies against CII. Mice were divided into four groups: 10 mg/kg IPI-145 starting on day 20, 50 mg/kg IPI-145 starting on day 20, 50 mg/kg IPI-145 starting on day 30, and vehicle. CIA mice that were treated with IPI-145 beginning on day 20 developed minimal signs of arthritis, with significant benefit observed at both doses (see Fig. 5A). Delayed therapy with IPI-145 modestly decreased joint swelling but had no effect on arthritis scores (Fig. 5, A and B). Histology scores on day 42 demonstrated a significant decrease in the early treatment regimen but not with delayed therapy compared with the vehicle treatment group (P < 0.05) (Fig. 5, C and D).

**Regulation of MMP Expression in CIA by PI3Kδ,γ Inhibition.** To evaluate the mechanism of action for IPI-145, the effect of the compound on synovial MMP expression was determined by quantitative real-time polymerase chain reaction. As with AA, expression of MMP-13 mRNA in ankle
joints was reduced by IPI-145 in the early treatment group compared with the vehicle treatment group (Fig. 6). Note that MMP-3 expression was also decreased by IPI-145 in this model. Effect of PI3K \( \delta \), \( \gamma \) Inhibition on the T-Cell Response in CIA. PI3K\( \gamma \) can potentially regulate T-cell development, proliferation, and cytokine production (Hirsch et al., 2000; Sasaki et al., 2000), whereas PI3K\( \delta \) is involved in T-cell differentiation and expansion (Oak et al., 2006). To determine the effect of PI3K\( \delta, \gamma \) inhibition on CII-specific T-cell responses in vivo, splenocytes from the 50 mg/kg IPI-145 day 20 treatment group and the vehicle treatment group were isolated and stimulated with the CII CB11 fragment. There was no significant effect of IPI-145 on CII-stimulated T-cell proliferation as determined by \(^{3}H\)thymidine incorporation, indicating that in vivo T-cell differentiation was not affected by exposure to the compound during the adaptive immune response (Fig. 7A). However, IL-17 production in response to CII peptide was significantly reduced in splenocytes from the IPI-145-treated group (\( n = 3 \) for each group; \( P < 0.05 \)) (Fig. 7B). Only very small amounts of interferon-\( \gamma \) and IL-4 were
produced in treated and control splenocytes and the PI3K inhibitors had no significant effect (data not shown).

**Effect of PI3Kδ,γ Inhibition on Anti-CII Antibodies in CIA.** PI3Kδ regulates antibody production from B cells, whereas PI3Kγ appears to play a minimal role in B cell function (Henley et al., 2008). To determine the effect of IPI-145 on B cells in vivo, the levels of anti-CII autoantibodies in CIA were measured in the vehicle and treatment groups. Serum concentrations of anti-CII total IgG were significantly lower in the 10 and 50 mg/kg IPI-145 early treatment groups than in the vehicle treatment ($P < 0.005$) or 50 mg/kg late treatment ($P < 0.05$) groups (Fig. 8A and B). Because anti-CII IgG2a is associated with binding to cartilage and complement fixation, we separately analyzed relative amounts of IgG2a. The anti-CII IgG2a/total IgG ratio was modestly decreased in the 10 mg/kg IPI-145 treatment and 50 mg/kg early treatment groups ($P < 0.005$ and $P < 0.05$, respectively) compared with the vehicle treatment group, whereas there was no difference of the ratio of IgG1 to total IgG in all groups (Fig. 8C). This result suggests that IPI-145 modestly suppresses the generation of pathogenic autoantibodies to CII, especially anti-CII IgG2a.

**Effect of PI3Kδ,γ on IgE Levels in CIA.** PI3Kδ is a negative regulator of IgE production in murine B cells that suppresses class switch recombination from IgG1 to IgE (Zhang et al., 2008). Therefore, PI3Kδ inhibition could potentially increase serum IgE levels. To evaluate this possibility, serum IgE concentrations were measured in the CIA mice. As shown in Fig. 8D, serum IgE was higher in the 50 mg/kg day 20 treatment group than in the vehicle treatment or the 10 mg/kg treatment groups. The IPI-145 50 mg/kg day 30 treatment group also has higher serum levels of IgE than the IPI-145
These results suggest that a PI3Kδ,γ inhibitor attenuates suppression of IgE class switching.

**Discussion**

Orally bioavailable small molecules could have significant advantages over protein therapeutics, and several classes of signal transduction inhibitors are being developed for RA. With the approval of a Janus kinase inhibitor in the United States, this alternative approach to biologics could become increasingly important. PI3K is another signaling pathway that is potentially relevant as a therapeutic target in inflammatory diseases because it plays a key role in both innate and adaptive immunity. Previous animal studies with PI3Kδ and PI3Kδ inhibition show that they independently regulate the development and progression of arthritis (Camps et al., 2005; Hayer et al., 2009; Gruen et al., 2010). Combined deficiency or
The inhibition of PI3K and PI3Kδ has striking therapeutic impact with near complete suppression of arthritis (Randis et al., 2008).

IPI-145 is a novel small molecule inhibitor of PI3Kδ,γ that reversibly inhibits both kinases. The $K_D$ values for IPI-145 are 0.023 nM for PI3Kδ, 0.24 nM for PI3Kγ, 1.56 nM for PI3Kβ, and 25.9 nM for PI3Ka, and the details of this compound are published elsewhere (Winkler et al., 2013). IPI-145 is much less active against PI3Ka and has a modest effect on PI3Kβ. Although the role of PI3Kβ is uncertain due to embryonic lethality in knockout mice, some data suggest that it can regulate neutrophil function (Kulkarni et al., 2011). Nevertheless, it is more likely that the pharmacologic activity observed in AA and CIA models is primarily due to an action on δ and γ isoforms.

In the present study, we show that a potent PI3Kδ,γ inhibitor, IPI-145, decreases disease severity in two models of chronic inflammatory arthritis (i.e., rat AA and mouse CIA). The induction of CIA requires both innate and adaptive immune responses through generation of pathogenic CII-specific T cells and production of CII-specific antibodies (Brand et al., 2003). On the other hand, AA requires T cell and neutrophils but appears to be independent of complement or B cells (Joe et al., 1999). Robust clinical and histopathologic evidence of improvement was observed in pretreatment regimens in both models. Delayed therapy for treatment of established disease was prominent in AA, whereas it was limited in CIA. The pathogeneses of the two models are quite distinct, with one being antibody and complement mediated and the other mediated by T cells and cannot be transferred by serum. Therefore, the differences likely are due to the role of PI3Kδ or δ in the two different mechanisms of disease, with a greater role in immune complex-mediated inflammation in CIA models. Because of the greater effect of innate immunity, the greater benefit in delayed disease might make sense in AA (Yanaba et al., 2007).

Cartilage and bone damage in RA is, in part, a consequence of an imbalance between excessive MMPs and inadequate
compensation of their inhibitors (Firestein, et al., 1991; Mohammed et al., 2003). MMP-3 and MMP-13 are highly expressed in pannus tissues of RA joints and they contribute to the irreversible joint destruction. IPI-145 reduced MMP gene expression in the two models and likely contributes to the observed protective effect. Decreased MMP mRNA could be due to a direct effect of PI3Kα inhibition on the signaling mechanisms required for MMP production. Alternatively, IPI-145 could secondarily decrease protease production by suppressing production of other inflammatory cytokines involved in induction of MMP, such as IL-1β or TNF. For example, TNF-mediated FLS activation is blocked by a PI3Kα inhibitor, suggesting that PI3Kα intersects with proinflammatory cytokine signaling (Bartok et al., 2012). This has also been observed in microglia in which MMP expression is PI3Kα dependent (Ito et al., 2007). Thus, the protective effect of IPI-145 could result from directly suppressing MMP expression or by decreasing cytokines that induce gene expression in FLSs.

We also evaluated whether IPI-145 blocks activation of its major downstream effector molecule, Akt. PI3K generates PI 3,4,5-triphosphate that binds to Akt at the plasma membrane, leading to activation of mammalian target of rapamycin complex 1 (Wu and Hu, 2010). The PI3K/Akt/mammalian target of rapamycin pathway plays a major role regulating cellular growth, survival, proliferation, and migration (Manning and Cantley, 2007). Our data confirm the biologic relevance of PI3Kα,γ blockade in vivo because Akt phosphorylation in joint tissues was decreased by IPI-145. Therefore, IPI-145 benefit is most likely due to direct modulation of the PI3K pathway.

The pathogenesis of CIA requires the generation of CII-specific T cells, which in turn, leads to the production of pathogenic antibodies. PI3Kα can clearly affect generation of autoreactive clones by regulating thymocyte maturation, T-cell proliferation and cytokine production by stimulation of the T-cell receptor, and immunologic synapse organization (Sasaki et al., 2000; Fruman and Cantley, 2002; Alcázar et al., 2007). PI3Kα also participates in T-cell maturation, differentiation, and trafficking by regulating lymph node homing receptor CD62L (Oak et al., 2006; Sinclair et al., 2008). Thus, altered T-cell responses could play a role in the beneficial effects of IPI-145. Despite the well defined role of PI3Kαs in T cells, there was no effect of IPI-145 on T-cell proliferative responses to CII peptide.

The proliferation data suggested that the IPI-145 might also control inflammatory arthritis through other mechanisms. One possibility is that the compound alters T-cell cytokine production, most notably IL-17, which is a critical cytokine in autoimmunity (Kunz and Ibrahim, 2009). Because PI3Kα regulates differentiation in T-cell subsets, we evaluated the T-cell cytokine response to CII peptide. Splenocytes from IPI-
PI3Kδ is also critical for B cell development, proliferation, and antibody production, and PI3Kδ-deficient B cells display reduced antibody production (Clayton et al., 2002; Okkenhaug et al., 2002). To determine the effect of IPI-145 on autoantibody production, we measured the serum level of anti-CII antibody subclasses in CIA. In the early treatment groups, anti-CII antibody production was reduced compared with control or delayed IPI-145 treatment. The onset and pathogene-

sis of CIA is associated with a predominance of pathogenic IgG2a because it binds to the cartilage and can fix complement. To determine the effect of IPI-145 on the predominance of anti-CII IgG2a, we confirmed that the anti-CII IgG2a/total IgG ratio was lower in the IPI-145 early treatment group than in the vehicle treatment group. Reduced anti-CII IgG2a production by IPI-145 is another factor that could contribute to decreased arthritis development and severity. It is not surprising, however, that delayed therapy has no effect. Previous studies using anti-CD20 antibodies to deplete B cells show that delayed therapy is ineffective in CIA and that benefit is only observed if B cells are depleted very early in the model (Yanaba et al., 2007).

IgE production, in contrast, was enhanced by PI3Kδ inhibition in CIA. Genetic or pharmaceutical blockade of PI3K δ110δ increases switch class recombination from IgG1 to IgE despite the decrease in autoreactive IgG2a antibodies (Zhang et al., 2008, 2012). Oral treatment with another PI3Kδ inhibitor, IC87114 (Sadhu et al., 2003), demonstrated a similar effect, implicating the δ isoform activity rather than γ in our studies (Zhang et al., 2008). Although the role of IgE in RA is not well defined, the presence of IgE anticitrullinated protein antibodies in RA patients supports a possible relationship of IgE and RA (Schuerwegh et al., 2010). Interestingly, phase I studies in humans have not shown an increase in IgE levels and suggest that this might be a rodent-specific phenomenon (Infinity Pharmaceuticals, unpublished observations).

The contribution of PI3Kγ inhibition to the efficacy of the inhibitor observed in the two animal models could be through multiple mechanisms, but most likely relates to its critical role in chemokine signaling (Li et al., 2000; Barbi et al., 2008, 2012). Oral treatment with another PI3Kδ inhibitor, IC87114 (Sadhu et al., 2003), demonstrated a similar effect, implicating the δ isoform activity rather than γ in our studies (Zhang et al., 2008). Although the role of IgE in RA is not well defined, the presence of IgE anticitrullinated protein antibodies in RA patients supports a possible relationship of IgE and RA (Schuerwegh et al., 2010). Interestingly, phase I studies in humans have not shown an increase in IgE levels and suggest that this might be a rodent-specific phenomenon (Infinity Pharmaceuticals, unpublished observations).

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