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A Novel Disease-Modifying Antirheumatic Drug, Iguratimod, Ameliorates Murine Arthritis by Blocking IL-17 Signaling, Distinct from Methotrexate and Leflunomide

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Iguratimod, a novel disease-modifying antirheumatic drug, which is now used in clinics in China and Japan, has been confirmed as a highly efficacious and safe drug for rheumatoid arthritis therapy. The antiarthritic mechanism of iguratimod, especially compared with that of the classical disease-modifying antirheumatic drugs, has not been elucidated. In this study, we conducted a comparative analysis of the antiarthritic effects of iguratimod and two reference drugs, methotrexate and leflunomide. We found that iguratimod dose dependently and potently inhibited arthritic inflammation of the synovium in collagen-induced arthritis and predominantly targeted IL-17 signaling. Consistent with its effects in vivo, iguratimod significantly suppressed the expression of various proinflammatory factors triggered by IL-17 in the cultured fibroblast-like synoviocytes. The inhibition of IL-17 signaling by iguratimod was further linked to a decrease in the mRNA stability of related genes and a reduction in phosphorylation of MAPKs. Iguratimod mainly targets Act1 to disrupt the interaction between Act1 and TRAF5 and IKKi in the IL-17 pathway of synoviocytes. Together, our results suggest that iguratimod yields a strong improvement in arthritis via its unique suppression of IL-17 signaling in fibroblast-like synoviocytes. This feature of iguratimod is different from those of methotrexate and leflunomide. This study may be helpful for further understanding the unique antiarthritic mechanism of iguratimod in patients with rheumatoid arthritis. The Journal of Immunology, 2013, 191: 4969–4978.

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heumatoid arthritis (RA) is a chronic systemic autoimmune disease that primarily targets the synovial membrane, cartilage, and bones of the hands, feet, and knees (1). The condition affects 1% of the population worldwide and often leads to disablement and a consequent reduction in the quality of life (2). Clinically, RA is characterized by synovial inflammation and hyperplasia (swelling), autoantibody production (rheumatoid factor and anti-citrullinated protein Ab), stiffness of the affected joints, cartilage and bone destruction (deformity), and systemic features (e.g., cardiovascular, pulmonary, psychological, and skeletal disorders) (3). Although the etiology of RA is unclear, the combination of a genetic predisposition and (4, 5) bacterial and viral infections (6) is thought to be involved in the initiating events of RA. Once the disease process is initiated, injury to the synovial vasculature occurs because of the dysregulation of T and B lymphocytes that results in immune responses directed against self-Ags (7). These cells infiltrate the synovium and are further activated to produce cytokines, which, in turn, activate various effector cells, such as neutrophils, monocytes/macrophages, dendritic cells, and mast cells (1, 8–10). The infiltrated inflammatory cells release cytokines, autoantibodies, and degradative enzymes, such as metalloproteinases, leading to cartilage destruction (11). Synovial hyperplasia leads to the formation of a tumor-like inflammatory tissue (“pannus”) that invaded the bone and degrades cartilage (12). The inflammation enhances the activity of resident fibroblast-like synoviocytes (FLSs) and osteoclasts, leading to bone erosion (7).

Among the effector cells involved in RA, Th17 cells are thought to play a critical role, both in RA patients and in a mouse model of collagen-induced arthritis (CIA) (13). IL-17A is the signature cytokine of the Th17 lineage (14, 15), which mediates proinflammatory effects via the induction of proinflammatory cytokines, chemokines, and metalloproteinases in a variety of cell types (16, 17). Upon ligand binding, IL-17 receptors recruit Act1 to form a complex that facilitates inflammatory responses (18). FLSs are the resident mesenchymal cells of the synovial joints and have been considered important in the pathogenesis of RA (19). FLSs normally function in joint homeostasis; however, the cells can secrete proinflammatory cytokines and synthesize specific chemokines, which may lead to the recruitment and activation of immune cells and to the ongoing inflammation and tissue destruction observed in RA patients (19, 20). Targeting the inflammatory signaling in FLSs may be a unique, effective approach for the treatment of RA.

Current remedies for RA include nonsteroidal anti-inflammatory drugs (NSAIDs) and disease-modifying antirheumatic drugs (DMARDs) (21–23). Only DMARDs can interfere with the dis-
ease process (24). Methotrexate is the most widely used small-molecular DMARD, and it continues to be used as the gold standard and the cornerstone of DMARD treatment regimens (2, 25–27). The latest addition to the DMARD family of drugs is leflunomide, an immunomodulatory agent involved in inhibiting de novo pyrimidine synthesis (28–30). Therapies targeting pyrimidine synthesis sometimes fail or produce only partial responses. All currently used DMARDs have issues, including limited efficacy and/or toxicity (2, 3, 31). Consequently, there is a growing interest in developing safe and effective treatments for RA. Iguratimod is an anti-inflammatory and immunomodulatory compound that has been developed as a novel DMARD. Although previous research indicated that it yielded improvements in several autoimmune models, such as CIA (32–34), the unique antiarthritic mechanism of iguratimod has not been elucidated. In this study, we selected methotrexate and leflunomide as reference drugs and conducted a comparative analysis of the antiarthritic effects of these DMARDs and iguratimod. This analysis revealed that iguratimod is a DMARD that is distinct from methotrexate and leflunomide. Iguratimod yields a strong improvement in arthritis via a unique mechanism targeting FLSs.

Materials and Methods

Mice

Specific pathogen–free, eight-week-old male DBA/1J mice were purchased from the Shanghai Experimental Animal Center, the Chinese Academy of Sciences. Animal welfare and experimental procedures were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and the related ethical regulations of Nanjing University. All efforts were made to minimize the animals’ suffering and to reduce the number of animals used.

Chemicals, Abs, and cytokines

Iguratimod was provided by Simcere Pharmaceutical (Nanjing, China), and its purity was >99%. Methotrexate and leflunomide were purchased from Sigma-Aldrich (St. Louis, MO). Bovine type II collagen (CII) was purchased from Chondrex (Seattle, WA). MTT and actinomycin D were purchased from Sigma-Aldrich (Burlingame, CA). Abs for Act1, matrix metalloproteinase 3 (MMP3), TRAF5, RANKL, and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Abs for p-p38, p-ERK, p-JNK, p-p65, and IKKi were purchased from Cell Signaling Technology (Beverly, MA). Recombinant cytokines IL-17, IFN-γ, and IL-1β and ELISA kits for IL-17 and IgG were purchased from R&D Systems (Minneapolis, MN). Cytometric bead array (CBA) cytokine assay kit was purchased from BD Biosciences (San Jose, CA). All other chemicals were purchased from Sigma-Aldrich.

Induction and assessment of CIA and drug administration

Male DBA/1J mice (7–8 wk) were immunized on day 0, at the base of the tail, with an intradermal injection of 100 μg CII emulsified in CFA (both from Chondrex). On day 21, mice were boosted with an injection of 100 μg CII dissolved in IFA (Sigma-Aldrich). Mice were examined daily and scored for arthritis severity, with each paw assigned a clinical score as follows: 0, normal; 1, erythema and mild swelling confined to the ankle joint and toes; 2, erythema and mild swelling extending from the ankle to the midfoot; 3, erythema and severe swelling extending from the ankle to the metatarsal joints; and 4, ankylosing deformity with joint swelling. Hind paw thickness was measured with an electronic plethysmometer (Paw Volume Meter). For treatment, iguratimod was administered intragastri- cally (i.g.) at 10, 30, or 100 mg/kg/d; leflunomide was administered i.g. at 10 mg/kg/d; and methotrexate was administered i.g. at 10 mg/kg/d every 2 d.

Histopathology

Paws for histological analysis were removed from mice and immediately fixed in 4% paraformaldehyde. The paws were decalcified in EDTA, embedded in paraffin, sectioned, and stained with H&E. The sections were scored to assess joint inflammation, on a scale of 0–4 under blinded

FIGURE 1. Improvement in murine CIA by oral administration of iguratimod, methotrexate, or leflunomide. Male DBA/1J mice were immunized with 100 μg of CII in CFA on day 0 and received a booster injection on day 21. Iguratimod and leflunomide were given i.g. each day beginning on day 0. Methotrexate was given i.g. once every 2 d. (A) The arthritis scores for CIA (mean ± SEM of seven mice/group). (B) Incidence of arthritis. (C) Photographs of representative paws from each CIA group. (D) The body weight changes in the DBA/1J mice were measured and calculated. Values are mean ± SEM of seven mice/group. (E) Representative radiographs of the hindpaws of the CIA mice were obtained on day 46 postimmunization. Arrows indicate areas of bone erosion. *p < 0.05, **p < 0.01 versus vehicle. Igu, Iguratimod; LFM, leflunomide; MTX, methotrexate.
conditions, according to the degree of hyperplasia in the synovial lining, mononuclear cell infiltration, and pannus formation.

**Immunohistochemistry**

Deparaffinized joint sections were subjected to Ag retrieval in 0.01 M citrate buffer solution. After blocking endogenous peroxidase activity in 3% H₂O₂, the sections were incubated with anti-MMP3 mAb, anti-RANKL mAb (both from Santa Cruz Biotechnology), or normal mouse IgG overnight at 4°C. Then the sections were rinsed and visualized by immunoperoxidase staining with a Real Envision Detection kit (GeneTech), according to the manufacturer’s instructions.

**Isolation of FLSs**

Synovial tissue was collected, minced, and incubated with 1 mg/ml type II collagenase (Sigma-Aldrich) in serum-free DMEM (Invitrogen) for 2 h at 37°C. After digestion, FLSs were washed extensively and cultured in DMEM supplemented with 10% FCS (Invitrogen) in a humidified 5% CO₂ atmosphere. After overnight culture, nonadherent cells were removed, and adherent cells were cultured in DMEM supplemented with 10% FCS. At confluence, cells were trypsinized, split at a 1:3 ratio, and recultured in medium. Synoviocytes from passages four through eight were used.

**Western blot**

Proteins were extracted in lysis buffer (30 mM Tris [pH 7.5], 150 mM sodium chloride, 1 mM PMSF, 1 mM sodium orthovanadate, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). The proteins were then separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were probed with Abs overnight at 4°C and then incubated with an HRP-coupled secondary Ab. Detection was performed using a LumiGLO chemiluminescent substrate system (Cell Signaling Technology).

**Cytokine measurement**

Cytokine levels were measured using a CBA cytokine assay kit (BD Biosciences) or ELISA kits (R&D Systems), according to the manufacturers’ instructions.

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**Quantitative real-time PCR**

Total RNA was extracted from cells using TRIzol reagent (Invitrogen). One microgram of RNA was reverse transcribed to cDNA. The mRNA expression was determined by real-time PCR using iQ SYBR Green Supermix (Bio-Rad). Mouse Actb gene was used as endogenous control for sample normalization. Results are presented as fold increases relative to the expression of mouse Actb. Sequences for PCR primer pairs are shown in Supplemental Table I.

**Statistics**

Data are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA, followed by the Student two-tailed t test. The p values < 0.05 were considered significant.

**Results**

Iguratimod attenuated the clinical signs of CIA similarly to methotrexate but had some advantages over methotrexate

Systemic arthritis was induced in male DBA/1J mice using CII in CFA, followed by a booster injection with CII in IFA on day 21 after primary immunization. Iguratimod and leflunomide were administered i.g. once a day beginning on day 0, and methotrexate was administered i.g. once every 2 d. We observed that the vehicle-treated mice responded to the immunization rapidly and developed arthritis within 1 wk, showing symptoms such as severe swelling, erythema, and joint rigidity in the hind paws. Leflunomide, at 10 mg/kg, yielded a slight decrease in arthritic scores and delayed the onset of arthritis. Treatment with methotrexate, at 10 mg/kg, demonstrated excellent antirheumatic properties, yielding reductions in arthritic scores and paw swelling. Iguratimod treatment delayed the onset of arthritis and attenuated the severity of CIA in a dose-dependent manner. The arthritic scores in the group treated with 10 mg/kg of iguratimod were lower than those of the

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**FIGURE 2.** The inhibitory effects of iguratimod, methotrexate, and leflunomide on proinflammatory cytokine and Ab production. Serum was obtained at peak clinical severity (day 46 postimmunization). (A) Cytokines in the serum were measured by CBA assay. Values are mean ± SEM of three mice/group. (B) Levels of total IgG and isotype-specific IgG2b anti-CII Abs after treatment with different DMARDs were determined using specific ELISA kits. Values are mean ± SEM of three mice/group. (C and D) Single-cell suspensions of regional lymph nodes obtained from drug- or vehicle-treated CIA mice on day 28 postimmunization were restimulated with denatured CII (50 μg/ml) for 72 h. (C) Supernatants were assessed by ELISA for the indicated cytokines. (D) Proliferation was examined by MTT assay. Values are mean ± SEM of three mice/group. For more details, see the legend for Fig. 1. *p < 0.05, **p < 0.01 versus normal; *p < 0.05, **p < 0.01 versus vehicle. Igu, Iguratimod; LFM, leflunomide; MTX, methotrexate.
leflunomide-treated mice and showed a 40–50% decrease compared with the scores of vehicle-treated mice. Treatment with 30 mg/kg of iguratimod resulted in a dramatic 80% decrease in the maximum arthritic scores, showing comparable efficacy to 10 mg/kg of methotrexate (Fig. 1). The hindpaw swelling correlated with the clinical scores (Supplemental Fig. 1A, 1B). In addition, we found that the administration of iguratimod starting from day 28 (after induction of CIA) also showed such efficacy (data not shown).

The body weight of each mouse was recorded every other day and expressed as the change in weight since arthritis induction (day 0). All of the mice gained weight at a similar rate before the onset of disease (days 0–21), with the exception of the methotrexate-treated mice, indicating that both leflunomide and iguratimod were well tolerated at the tested doses, whereas methotrexate caused a large reduction in body weight (Fig. 1D). Some adverse events were detected in the mice treated with 10 mg/kg of methotrexate, such as loss of appetite and lack of movement.

We detected bone erosion, articular destruction, and joint displacement upon radiographic evaluation of the forepaws and hindpaws of the vehicle-treated mice on day 46 postimmunization (Fig. 1E). In comparison, iguratimod, at 10 mg/kg, protected the mice from bone erosion and joint destruction, and almost no bone erosion was observed in the groups given 30 or 100 mg/kg of iguratimod or 10 mg/kg of methotrexate. No significant protection was observed with 10 mg/kg of leflunomide.

Iguratimod showed a different inhibition profile against inflammatory cytokines and Abs compared with classical DMARDs

To elucidate the mechanisms underlying the improvement of CIA after iguratimod treatment, we examined the serum concentrations of different proinflammatory cytokines in CIA mice. Iguratimod, at 10, 30, and 100 mg/kg, inhibited the production of IL-17, IFN-γ, and IL-6, which was comparable to the effects of methotrexate. Iguratimod showed mild inhibition against TNF-α and had no effect on IL-10. Methotrexate, at 10 mg/kg, showed strong inhibition against the production of these cytokines, whereas leflunomide (10 mg/kg) had either a mild effect or no effect. With regard to the serum levels of total IgG or isotype-specific IgG2b, iguratimod (30 and 100 mg/kg) and methotrexate (10 mg/kg) resulted in significant inhibition, whereas leflunomide (10 mg/kg) did not (Fig. 2B). There was no significant difference between the iguratimod- and vehicle-treated CIA mice with regard to IL-17 production (Fig. 2C) and cell proliferation (Fig. 2D) of the isolated lymphocytes at day 28 in response to denatured type II collagen stimulation.

Iguratimod resulted in a slight inhibition of T cell proliferation induced by Con A (Supplemental Fig. 1C). A similar result was observed in the T cells treated with methotrexate and leflunomide. We examined whether these three DMARDs could induce apoptosis of lymphocytes. At concentrations up to 10 μM, neither iguratimod nor leflunomide affected the apoptotic cell counts. Cell apoptosis was detected in methotrexate-treated lymphocytes, which may be responsible for the adverse effects of this drug in mice (Supplemental Fig. 1D). Given the reduction in Abs in CIA mice, it seems likely that all three drugs could regulate B cell function. We found that iguratimod dose dependently inhibited the proliferation of LPS-stimulated B cells (Supplemental Fig. 1E). With methotrexate or leflunomide, the proliferation was moderately inhibited at all concentrations from 0.1 to 10 μM, suggesting that the inhibition is due to drug cytotoxicity. Iguratimod caused a concentration-dependent reduction in the expression of the B cell activation marker CD23 (Supplemental Fig. 1F). Iguratimod and leflunomide yielded moderate inhibition of IgG and IgM levels in the culture.

![FIGURE 3. Reduced inflammatory cells in the arthritic joints after iguratimod, methotrexate, or leflunomide treatment. Arthritic joints from CIA mice were harvested on day 46 postimmunization. (A and B) Inflammatory infiltration scores were assessed by H&E staining. Original magnification ×100. Values are mean ± SEM of four mice/group. (C) Expression levels of Cd4, Cd8, Igam, and Ly6g mRNA in CIA mice were determined by quantitative real-time PCR. Values are mean ± SEM of three mice/group. For more details, see the legend for Fig. 1. *p < 0.05, **p < 0.01 versus normal; *p < 0.05, **p < 0.01 versus vehicle. Igu, Iguratimod; LFM, leflunomide; MTX, methotrexate.]
supernatants in a dose-dependent manner, whereas all concentrations (0.1–10 μM) of methotrexate strongly reduced IgG and IgM production (Supplemental Fig. 1G, 1H).

Iguratimod inhibited inflammatory cell infiltration into arthritic joints as strongly as methotrexate

Histological analysis of the inflamed hindpaws showed synovial hyperplasia, joint destruction, and inflammatory cell infiltration in vehicle-treated mice, with a high inflammation score of 5.75 ± 0.85 (Fig. 3A, 3B). Iguratimod, at a dose of 30 mg/kg, significantly reduced the inflammation score to 1.00 ± 0.57 (p < 0.01). The inhibitory effect was equivalent to that of methotrexate treatment (Fig. 3A, 3B). We analyzed the joint infiltrates using real-time RT-PCR and confirmed that T cells, macrophages, and neutrophils were recruited into arthritic joints, based on higher expression levels of Cd4, Cd8, Igum, and Ly6g. Such infiltrates were markedly reduced after treatment with iguratimod or methotrexate (Fig. 3C). Leflunomide showed only a moderate or no effect on inflammatory cell infiltration (Fig. 3).

Iguratimod predominantly suppressed expression levels of cytokines and chemokines involved in IL-17 signaling in arthritic joints, which was distinct from the modes of action of methotrexate and leflunomide

The infiltration of T cells and neutrophils into the joint tissues is a predominant feature of RA. FLSs may function to facilitate cell recruitment and retention through the expression of specific proinflammatory genes. To further determine how iguratimod inhibits inflammatory cell infiltration, we examined the expression of an array of chemokines in the arthritic joints. The results showed that the expression of certain inflammatory cytokines and chemokines involved in IL-17 signaling, including Ccl2, Cxcl1, Cxcl2, and Il-6, was greatly reduced after iguratimod or methotrexate treatment (Fig. 4A). Iguratimod significantly suppressed the expression of Ccl2 and the production of RANKL in joint tissues, as demonstrated by the immunohistochemical assay (Fig. 4B). Methotrexate inhibited the expression of Cxcl9 and Cxcl10 in IFN-γ signaling, whereas iguratimod exhibited a mild inhibition of expression (Fig. 4A). In the above cases, leflunomide signifi-

FIGURE 4. Decreased expression of proinflammatory factors in joints after iguratimod, methotrexate, or leflunomide treatment. (A) Total RNA was extracted from the ankle and metatarsal joints of three individual mice from the naive group, the vehicle-treated group, and the drug-treated group on day 46 postimmunization. The mRNA levels of Ccl2, Cxcl1, Cxcl2, Il6, Cxcl9, and Cxcl10 were measured by quantitative real-time PCR. Values are mean ± SEM of three mice/group. (B) Hindpaw sections obtained from mice with CIA on day 46 postimmunization were stained with anti-MMP3 and anti-RANKL in an immunohistochemical assay. A representative of three independent experiments is shown. Original magnification ×100. For more details, see the legend for Fig. 1. *p < 0.05 versus normal, *p < 0.05 versus vehicle. Igu, Iguratimod; LFM, leflunomide; MTX, methotrexate.
significantly reduced the expression of IL6 and CXCL10 but did not influence other chemokines or cytokines.

Iguratimod, but not methotrexate or leflunomide, specifically suppressed IL-17 signaling in FLSs

To further confirm the in vivo findings that iguratimod mainly inhibited inflammatory gene (cytokines, chemokines, and matrix metalloproteinases) expression induced by IL-17, rather than by IFN-γ, we analyzed the expression of several proinflammatory genes by quantitative real-time PCR in primary FLSs after treatment with IL-17A, IFN-γ, or IL-1β. We found that the expression of CCL2, CXCL1, CXCL2, IL6, TNF, and CSF3 induced by IL-17 was significantly reduced in the presence of iguratimod, whereas no such inhibition was detected in the methotrexate and leflunomide groups (Fig. 5A). Iguratimod did not show any influence on these genes under the stimulation of IL-1B (Supplemental Fig. 2A).

The IFN-γ–induced expression of CXCL9 and CXCL10 was not changed by iguratimod treatment (Supplemental Fig. 2B). Because IL-17 synergizes with TNF-α with regard to inflammatory cytokine production, we assessed the effects of iguratimod on the induction of chemokines expression induced by TNF-α plus IL-17. We found that iguratimod significantly inhibited the expression of chemokines, such as CCL2 and CXCL1, induced by TNF-α plus IL-17 (Supplemental Fig. 2C). We then measured the activation of MAPKs by Western blot analysis. We examined the effect of iguratimod on IL-17–treated cells incubated with the compound for 2 h prior to stimulation with IL-17. We found that iguratimod treatment resulted in a dose- and time-dependent decrease in the levels of p-p38 and p-JNK, as well as a mild reduction in p-ERK, after stimulation with IL-17 (Fig. 5B, 5C). Meanwhile, iguratimod showed the same effect on IL-17–stimulated cells treated with compounds for 6 h prior to IL-17 stimulation (data not shown). In addition, we defined the effects of iguratimod on the NF-κB pathway. We performed Western blot analysis and NF-κB reporter gene assays and found that iguratimod did not affect the NF-κB pathway (Supplemental Fig. 3).

To explore the effect of iguratimod on mRNA stability of the chemokines, FLSs were treated with vehicle or 10 μM iguratimod for 4 h, followed by stimulation with TNF-α for 1 h to promote transcription. The cells were treated with actinomycin D (to block transcription) and IL-17 for an additional 4 h. As shown in Fig. 5C, IL-17 stabilized the mRNA levels of CCL2 and CXCL11 in the presence of actinomycin D. These genes decayed rapidly with iguratimod treatment (Fig. 5D).

Iguratimod targeted Act1 to disrupt the interaction of Act1 with TRAF5 and IKKi in IL-17 signaling in FLSs

Then we investigated whether iguratimod regulates Act1–adapted complex formation in response to IL-17 stimulation. IL-17 stimulation induced the interaction of Act1 with IL-17R, TRAF5, TRAF6, and IKKi in primary FLSs. We found that iguratimod disrupted Act1–TRAF5 and Act1–IKKi interactions but not the Act1–TRAF6 and Act1–IL-17R associations (Fig. 6A). When we used Abs to TRAF5 and TRAF6 for the immunoprecipitation of the lysates of FLSs, we detected a decreased level of Act1 in the immune complex of TRAF5, but not of TRAF6, after iguratimod treatment (Fig. 6B). Colocalization of Act1 and TRAF5 was markedly reduced after exposure to 10 μM of iguratimod (Fig. 6C).

FIGURE 5. Suppressive effects of iguratimod, methotrexate, and leflunomide on the expression of chemokines in vitro. (A) Primary FLSs were incubated with the three DMARDs and stimulated by 50 ng/ml of IL-17. Sixteen hours later, the expression levels of chemokines were determined by quantitative real-time PCR. Values are mean ± SEM of three independent experiments. (B) FLSs were incubated with iguratimod (1, 3, 10 μM) and methotrexate (MTX, 10 μM) for 2 h, and the cells were stimulated with 50 ng/ml of IL-17 for 10 min. Total proteins were extracted, and immunoblot analysis was performed. A representative of three independent experiments is shown. (C) FLSs were incubated with 10 μM of iguratimod for 2 h, and the cells were stimulated with 50 ng/ml of IL-17 for the indicated time. Total proteins were extracted, and immunoblot analysis was performed. A representative of three independent experiments is shown. (D) FLSs were treated with vehicle or 10 μM of iguratimod for 6 h, and the cells were stimulated with 10 ng/ml of TNF-α for 1 h to promote transcription. The cells were treated with 5 μg/ml of actinomycin D (ActD) and 50 ng/ml of IL-17 for the indicated time.

The mRNA levels of CCL2 and CXCL11 were determined by real-time PCR. *p < 0.05, **p < 0.01 versus IL-17 alone.
All of the above observations prompted us to identify the potential targets of iguratimod in FLSs. For this purpose, biotin-tagged iguratimod (hereafter referred to as “biotin-iguratimod,” Fig. 7A) was designed to be a useful chemical probe. As shown in Fig. 7B, biotin-iguratimod effectively pulled down Act1, but not TRAF5; this binding was competitively inhibited by higher concentrations of unlabeled iguratimod, indicating that iguratimod directly targeted Act1. Moreover, immunofluorescence staining with an Ab against Act1 and streptavidin-FITC also demonstrated that biotin-iguratimod colocalized with Act1 in the cytoplasm of FLSs (Fig. 7C). In addition, Act1 overexpression desensitized cells to iguratimod, further supporting the notion that iguratimod’s molecular target is Act1 (Fig. 7D).

Discussion
Traditionally, RA has been treated with NSAIDs, glucocorticoids, and DMARDs (24). Only DMARDs can impede the inflammatory and destructive processes of RA. Many DMARDs reduce pain, swelling, and the destructive progression of the disease. If treatment with a DMARD is efficacious, patients often do not need
FIGURE 7. Iguratimod targets Act1. (A) Chemical structure of biotin-iguratimod. (B) IL-17-induced FLS lysates were incubated with biotin-iguratimod (Igu) or biotin in the absence or presence of a 10- or 20-fold excess of unlabeled iguratimod, followed by pull-down with avidin resin. The precipitates were resolved by SDS-PAGE, and the gel was detected by Western blotting for Act1 and TRAF5. (C) After being treated or not with 20 μM biotin-iguratimod for 2 h, FLSs were incubated with IL-17 for 15 min and then stained with an Ab (anti-Act1; red) for Act1 and streptavidin-FITC (green) for biotin, followed by counterstaining with DAPI (original magnification ×100). (D) Mouse embryonic fibroblast cells were transfected with empty vector or vector encoding HA-tagged Act1. After transfection for 24 h, cells were treated with iguratimod (3, 10 μM) for 2 h, and cells were stimulated with 50 ng/ml of IL-17 for 15 min. Total proteins were extracted, and immunoblot analysis was performed. A representative of three independent experiments is shown.

Iguratimod targets Act1. (A) Chemical structure of biotin-iguratimod. (B) IL-17-induced FLS lysates were incubated with biotin-iguratimod (Igu) or biotin in the absence or presence of a 10- or 20-fold excess of unlabeled iguratimod, followed by pull-down with avidin resin. The precipitates were resolved by SDS-PAGE, and the gel was detected by Western blotting for Act1 and TRAF5. (C) After being treated or not with 20 μM biotin-iguratimod for 2 h, FLSs were incubated with IL-17 for 15 min and then stained with an Ab (anti-Act1; red) for Act1 and streptavidin-FITC (green) for biotin, followed by counterstaining with DAPI (original magnification ×100). (D) Mouse embryonic fibroblast cells were transfected with empty vector or vector encoding HA-tagged Act1. After transfection for 24 h, cells were treated with iguratimod (3, 10 μM) for 2 h, and cells were stimulated with 50 ng/ml of IL-17 for 15 min. Total proteins were extracted, and immunoblot analysis was performed. A representative of three independent experiments is shown.

Because histological analysis showed that iguratimod dose dependently reduced inflammatory infiltration with an equivalent inhibitory effect to that of methotrexate (Fig. 3), we explored an array of cytokines and chemokines in arthritic joints, including those involved in IL-17 and IFN-γ signaling. The results showed that Ccl2, Cxcl1, Cxcl2, and Il6, which are IL-17–induced chemokines, were greatly reduced by treatment with iguratimod or methotrexate but not with leflunomide. Iguratimod significantly suppressed MMP3 and RANKL expression in joint tissues. Iguratimod showed virtually no inhibition of the Cxcl9 or Cxcl10 expression involved in IFN-γ signaling, whereas methotrexate inhibited the expression of these two chemokines (Fig. 4). These findings suggest that iguratimod primarily inhibits chemokine expression induced by IL-17 rather than by IFN-γ, which is the major characteristic of iguratimod that distinguishes it from methotrexate and leflunomide.

Iguratimod targets Act1. (A) Chemical structure of biotin-iguratimod. (B) IL-17-induced FLS lysates were incubated with biotin-iguratimod (Igu) or biotin in the absence or presence of a 10- or 20-fold excess of unlabeled iguratimod, followed by pull-down with avidin resin. The precipitates were resolved by SDS-PAGE, and the gel was detected by Western blotting for Act1 and TRAF5. (C) After being treated or not with 20 μM biotin-iguratimod for 2 h, FLSs were incubated with IL-17 for 15 min and then stained with an Ab (anti-Act1; red) for Act1 and streptavidin-FITC (green) for biotin, followed by counterstaining with DAPI (original magnification ×100). (D) Mouse embryonic fibroblast cells were transfected with empty vector or vector encoding HA-tagged Act1. After transfection for 24 h, cells were treated with iguratimod (3, 10 μM) for 2 h, and cells were stimulated with 50 ng/ml of IL-17 for 15 min. Total proteins were extracted, and immunoblot analysis was performed. A representative of three independent experiments is shown.
or leflunomide-treated FLSs (Fig. 5A). In addition, it was reported that when a single 10-mg/kg dose of iguratimod was given to the rats i.g., the mean maximum plasma concentration of iguratimod for the six rats was 14.5 µg/ml (≈39 µM) by HPLC assay (44). According to such pharmacokinetics characteristics of iguratimod, the drug concentrations (1–10 µM) that we used on FLSs were much lower than the blood drug level in mice when iguratimod (100–100 mg/kg/d) was given orally for 46 d. Thus, the effects of IL-17 on FLSs in vitro are relevant to the efficacy in mice. These findings suggest that FLSs may be the primary target of iguratimod but not of methotrexate or leflunomide. The activation of p38, ERK, and JNK, but not of p65, induced by IL-17 in the synoviocytes was inhibited by iguratimod to different degrees (Fig. 5B, 5C). These results further verified the activity of iguratimod specifically against IL-17 signaling, which is an activity unique to iguratimod.

Considering that iguratimod did not affect NF-κB activation, we supposed that iguratimod may regulate the IL-17–mediated stabilization of chemokine mRNA, as previously reported (45). As shown in Fig. 5D, IL-17 stabilized the mRNA levels of Ccl2 and Cxcl1 in the presence of actinomycin D, and iguratimod caused decreased expression of these genes. These findings indicate that iguratimod restricts IL-17–mediated activation of MAPKs and stabilization of chemokine-encoding mRNAs, which are characteristics distinguishing it from classical DMARDs.

Act1, the key adaptor protein in IL-17 signaling, is an absolute requirement for the development of joint pathology in CIA (41). IL-17 can activate NF-κB and MAPKs in an Act1–TRAF6– and Act1–TRAF5–dependent manner, respectively. TRAF5 and TRAF6 function downstream of Act1 to mediate the stability of chemokine-encoding mRNA and the activation of NF-κB, respectively (46). Because iguratimod regulated the IL-17–induced activation of MAPKs and stabilization of chemokine-encoding mRNA, which involves TRAF5 and IKKı (47, 48), we examined whether iguratimod inhibited the Act1-adapted complex formation in response to IL-17 stimulation. Although IL-17 stimulation induced the interaction of Act1 with TRAF5, TRAF6, and IKKı in primary FLSs, we found that iguratimod specifically disrupted Act1–TRAF5 and Act1–IKKı interactions but not the Act1–TRAF6 association (Fig. 6A). When we used Abs to TRAF5 and TRAF6 for the immunoprecipitation of the lysates of FLSs, we found that iguratimod decreased the level of Act1 in the immune complex of TRAF5 but not of TRAF6 (Fig. 6B). In addition, the colocalization of Act1 and TRAF5 was markedly reduced after exposure to 10 µM iguratimod (Fig. 6C). Taken together, these data suggest that iguratimod blocks the interaction of Act1 with TRAF5 and IKKı, which may explain its inhibitory effect on the IL-17 pathway.

Using biotin-iguratimod as a probe, we found that iguratimod targets Act1 but not TRAF5. According to the immunofluorescence study, the binding occurs in the cytoplasm. In addition, Act1 overexpression desensitized cells to iguratimod (Fig. 7). These results suggest that Act1 is a molecular target of iguratimod. Further study on the molecular basis for iguratimod and Act1 is in progress.

To our knowledge, our data demonstrate for the first time that iguratimod can block the IL-17 pathway in FLSs by targeting Act1 to disrupt its interactions with TRAF5 and IKKı, which is linked to the effective treatment of CIA. The novel DMARD, iguratimod, which is now used in clinics in China and Japan, has been confirmed as a highly effective and safe drug for therapy that elicits only slight, attenuated systemic immune responses. This study identified a specific mechanism of iguratimod that is different from the mechanisms of the classical DMARDs, methotrexate and leflunomide, which may remedy shortcomings in the treatment of RA.


