RN486, a Selective Bruton’s Tyrosine Kinase Inhibitor, Abrogates Immune Hypersensitivity Responses and Arthritis in Rodents

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ABSTRACT

Genetic mutation and pharmacological inhibition of Bruton’s tyrosine kinase (Btk) both have been shown to prevent the development of collagen-induced arthritis (CIA) in mice, providing a rationale for the development of Btk inhibitors for treating rheumatoid arthritis (RA). In the present study, we characterized a novel Btk inhibitor, 6-cyclopropyl-8-fluoro-2-(2-hydroxymethyl-3-{1-methyl-5-[5-(4-methyl-piperazine-1-yl)-pyridin-2-ylamino]-6-oxo-1,6-dihydro-pyridin-3-yl}-pyrenyl)-2H-isoquinolin-1-one (RN486), in vitro and in rodent models of immune hypersensitivity and arthritis. We demonstrated that RN486 not only potently and selectively inhibited the Btk enzyme, but also displayed functional activities in human cell-based assays in multiple cell types, blocking Fcγ receptor cross-linking-induced degranulation in mast cells (IC50 = 2.9 nM), Fcγ receptor engagement-mediated tumor necrosis factor α production in monocytes (IC50 = 7.0 nM), and B cell antigen receptor-induced expression of an activation marker, CD69, in B cells in whole blood (IC50 = 21.0 nM). RN486 displayed similar functional activities in rodent models, effectively preventing type I and type III hypersensitivity responses. More importantly, RN486 produced robust anti-inflammatory and bone-protective effects in mouse CIA and rat adjuvant-induced arthritis (AIA) models. In the AIA model, RN486 inhibited both joint and systemic inflammation either alone or in combination with methotrexate, reducing both paw swelling and inflammatory markers in the blood. Together, our findings not only demonstrate that Btk plays an essential and conserved role in regulating immunoreceptor-mediated immune responses in both humans and rodents, but also provide evidence and mechanistic insights to support the development of selective Btk inhibitors as small-molecule disease-modifying drugs for RA and potentially other autoimmune diseases.

Introduction

Rheumatoid arthritis (RA) is an autoimmune joint disease characterized by chronic synovial inflammation and progressive joint destruction. The disease is often associated with the appearance of autoantibodies in both blood and inflamed tissues. Genetic and environmental factors contribute to the development of RA, and the immune system plays a critical role in its pathogenesis. Recent studies have shown that Bruton’s tyrosine kinase (Btk) is involved in the regulation of immune responses, and inhibitors of Btk have shown promise in preclinical models of RA.

RN486 is a selective Btk inhibitor characterized by an IC50 of 2.9 nM in human mast cell degranulation assays and 7.0 nM in monocyte tumor necrosis factor α production. In vivo, RN486 effectively prevented type I and type III hypersensitivity responses in mice and produced robust anti-inflammatory and bone-protective effects in mouse collagen-induced arthritis (CIA) and rat adjuvant-induced arthritis (AIA) models. These findings support the development of selective Btk inhibitors as potential therapeutic agents for RA and other autoimmune diseases.
ITAMs to phospholipase C-\(\gamma\) (Btk) from the sarcoma kinase, Syk, and Tec kinase way regulated by nonreceptor tyrosine kinases including Nimmerjahn and Ravetch, 2008). ITAMs act to integrate immunoreceptors containing an intracellular immunoreceptor tyrosine-based activation motif (ITAM) (Kurosaki, 2000; Nimmerjahn and Ravetch, 2008). ITAMs act to integrate diverse antigen- or Fc-specific signals into a common pathway regulated by nonreceptor tyrosine kinases including Lyn, spleen tyrosine kinase (Syk), and Bruton’s tyrosine kinase (Btk) from the sarcoma kinase, Syk, and Tec kinase families. These kinases relay the signals sequentially from ITAMs to phospholipase C-\(\gamma\)2 (PLC-\(\gamma\)2) (Kurosaki et al., 2000) and thus play a critical and nonredundant role in the signal transduction of BCR and FcR. Consequently, loss-of-function mutation in the Btk gene results in severe B cell immunodeficiency and impaired FcR function in both patients with X-linked agammaglobulinemia and mutant mice with X-linked immunodeficiency (Thomas et al., 1993; Conley et al., 1994).

Btk also regulates the signaling of other ITAM-containing receptors or adaptors, e.g., DNAX-activating protein of 12 kDa (DAP12) (Koga et al., 2004). DAP12 functions as an adaptor protein for multiple receptors, including macrophage colony-stimulating factor 1 receptor (CSF1R) and myeloid DAP12-associated lectin-1 receptor. Both receptors have been implicated in the development of immune arthritis (Huang et al., 2009; Joyce-Shaikh et al., 2010). Also importantly, CSF1R, along with FcR gamma chain, provides a secondary, but indispensable, signal for RANKL to promote osteoclast differentiation and, subsequently, bone resorption (Koga et al., 2004).

Together, Btk and Syk regulate the signal transduction of ITAM-containing receptors or adaptors that are critical for autoantibody production, effector function, and osteoclast differentiation. Therefore, pharmacological inhibition of these enzymes may affect multiple steps in the pathogenesis of RA and represent a useful approach for the treatment of the disease. Indeed, R788 (fostamatinib), a first-generation Syk inhibitor, has been shown to have benefits in both animal models of immune arthritis and patients with RA (Weinblatt et al., 2010), supporting the concept that inhibitors of Syk and its downstream kinase Btk may have utilities as RA therapeutics. Indeed, emerging evidence demonstrates that the inhibition of Btk in mice confers resistance to CIA (Höögberg et al., 2010; Di Paolo et al., 2011; Liu et al., 2011), similarly to Btk mutation (Jansson and Holmdahl, 1993).

In the present study, we characterized a novel, potent, and selective reversible Btk inhibitor, 6-cyclopropyl-8-fluoro-2-(2-hydroxymethyl-3-(1-methyl-5-[(4-methyl-piperazin-1-yl)-pyridin-2-ylamino]-6-oxo-1,6-dihydro-pyridin-3-yl)-phenyl)-2H-isooquinolin-1-one (RN486), in multiple mechanistic as well as disease-related cellular assays and animal models. We demonstrated that RN486 inhibited immune responses mediated by two important immunoreceptors, BCR and FcR, in both human cells and rodents. It is noteworthy that RN486 produced dose-dependent efficacy in both CIA and AIA, with evidence for substantial efficacy in combination with low-dose methotrexate. These findings support the emerging concept that Btk plays a mandatory role in regulating immunoreceptor signaling in both humans and rodents and suggest the potential utility of selective Btk inhibitors for the treatment of RA and other autoimmune diseases.

### Materials and Methods

#### Enzymatic Activity and Binding Assays

**Btk Enzymatic Activity Assay.** A fluorescence-based kinase assay (Caliper Life Sciences, Hopkinton, MA) was used to measure Btk kinase activity and its inhibition by RN486. In the assay, test compound was incubated with a human recombinant Btk (10 nM), ATP (100 \(\mu\)M), and a fluorescent-labeled peptide substrate (15 \(\mu\)M) at 30°C for 15 min. The reaction was then stopped by the addition of a termination buffer, and reaction product was quantified by using a Desktop Profiler 4 sipper (Caliper Life Sciences).

**Time-Resolved Fluorescence Resonance Energy Transfer-Based Competitive Binding Assay.** The assay was used to determine the ability of RN486 to competitively inhibit binding of a substrate to Btk. It was adapted from a standard “one step, mix-and-read, time-resolved FRET binding assay” from Invitrogen (Carlsbad, CA). In brief, BTK-BioEase, a biotinylated BTK, was conjugated to a FRET donor, europium, through an incubation with Eu-streptavidin (PerkinElmer Life and Analytical Sciences, Waltham, MA) for 1 h in a buffer containing 20 mM HEPES, pH 7.15, 0.1 mM dithiothreitol, 10 mM MgCl2, and 0.5 mg/ml bovine serum albumin. BTK-Eu conjugate (0.1 nM) was then incubated with KT-178, a kinase tracer and FRET acceptor from Invitrogen, and RN486 or vehicle (DMSO) overnight at 15°C. Photons emitted from both the donor (620 nm) and acceptor (FRET; 665 nm) were measured by using a BMG Pherastar Fluorescent plate reader (BMG Labtech GmbH, Offenberg, Germany). Ratio between the 620- and 665-nm signals was calculated to determine the ability of RN486 to competitively inhibit binding of a substrate to Btk.

**Kinomescan Selectivity Assay.** Selectivity of RN486 for Btk over non-Btk kinases was determined by testing the compound at a single 10 \(\mu\)M concentration against a panel of 396 kinases at Ambit Biosciences (San Diego, CA) by using a high-throughput Kinomescan based on ATP free competitive binding (Fabian et al., 2005). RN486 was further titrated against individual kinases that were inhibited by >85% at 10 \(\mu\)M to determine the corresponding \(K_i\) values. Fold of selectivity for Btk over other kinases (kinase X) was defined as \(K_i^{\text{Btk}} / K_i^{\text{X}}\).

#### Cell-Based Functional Assays

**Detection of Phospho-BTK and Phospho-PLC-\(\gamma\)2 in B Cells.** Human B cells were purified by using a RosetteSep Human B Cell Enrichment Cocktail (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer’s instruction. A total of 1 × 10^6 cells were pretreated with DMSO or 1 \(\mu\)M RN486 for 15 min followed by 5-min stimulation with goat anti-human IgM (10 \(\mu\)g/ml; Southern Biotechnology Associates, Birmingham, AL). B cells were pelleted and lysed in M-PER Mammalian Protein Extraction buffer (Thermo Fisher Scientific, Waltham, MA). Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred into polyvinylidene
diffuorol membrane and then blotted with mouse anti-BTK (BD Biosciences, San Jose, CA), mouse antiphospho-BTK (Tyr551) (gift from Owen Witte, University of California, Los Angeles, CA) or rabbit anti-PLCγ2 antibody or rabbit antiphospho-PLCγ2 (Tyr1217) (both from Cell Signaling Technology, Danvers, MA), and corresponding secondary antibodies.

Ramos Calcium Influx Fluorometric Imaging Plate Reading Assay. Ramos cells (CRL-1596; American Type Culture Collection, Manassas, VA) were seeded in an assay plate, 125,000 cells per well, in phenol red-free RPMI 1640 medium containing 2% heat-inactivated fetal bovine serum (Invitrogen), and loaded with a BD calcium dye for 1 h (BD Biosciences). Next, cells were treated with either RN486 or vehicle for 30 min in the dark before the plate was transferred to a FLIPR Tetra (Molecular Devices, Sunnyvale, CA). Immediately after the transfer and a 10-s recording of baseline fluorescence, cells were stimulated with a mouse anti-human IgM antibody (αIgM, 10 μg/ml, clone M2E6) (Antibody Solutions, Mountain View, CA) for 150 s, during which fluorescence signal was monitored and recorded. Difference between the signal and that at baseline, designated adjusted relative fluorescence unit, was calculated by using a custom Excel (Microsoft, Redmond, WA) template to determine αIgM-induced calcium influx and its inhibition by RN486.

B Cell CD69 Assay in Human Whole Blood. Heparinized blood was freshly collected from healthy volunteers, aliquoted onto an assay plate (100 μl/well), and incubated with test compound for 30 min before the addition of goat Fab(ab)′2 anti-human IgM antibody (αIgM; final concentration 50 μg/ml) (Biotechnology Association Associates). After a 20-h incubation, samples were added to two fluochrome-labeled mouse anti-human antibodies, one for CD20 and the other for CD69 (BD Biosciences), and further incubated for 30 min. Samples were then mixed with a lysis buffer (BD Biosciences) to remove red blood cells and washed with phosphate-buffered saline containing 2% fetal bovine serum. Immediately after the wash, fluorescence signal for CD69 or CD20 (B cells)-positive cells was acquired and analyzed by using an LSR II flow cytometer (BD Biosciences) and FlowJo software (Treestar, Ashland, OR).

FcyR-Mediated TNFα Production Assay in Human Monocytes. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation at 2200 rpm from heparinized blood freshly collected from healthy volunteers. PBMCs (1 × 106 cells/well) were cultured in a 96-well plate for 1 to 2 h to allow monocytes to adhere. After the removal of nonadherent cells, monocytes were incubated with test compound alone for 30 min and then with human IgG-coated beads (0.25 mg/ml IgG; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) or for 4 h. TNFα in supernatants was determined by enzyme-linked immunosorbent assay (BD Biosciences).

Anti-4-Hydroxy-3-Nitrophenylacetyl Hapten IgE-Induced Histamine Release in Human Mast Cells. One million human cord blood-derived CD34+ hematopoietic stem cells (ALLCells, LLC, Emeryville, CA) were cultured in a serum-free StemPro-34 complete medium (Invitrogen) with supplements, stem cell factor (100 ng/ml), and IL-6 (50 ng/ml). IL-3 (10 ng/ml) was also included during the first week to support cell differentiation (all cytokines from R&D Systems, Minneapolis, MN). After 8 weeks of culture, cells were stimulated with IL-4 (10 ng/ml) for 5 days. By then, most cells (90%) were differentiated into c-kit- and FcεRI-positive mast cells.

For measuring histamine release, 0.1 million mast cells were seeded in 96-well round-bottom culture plates. Cells were sensitized with 0.1 μg/ml anti-NP IgE (Serotec, Raleigh, NC) overnight at 37°C. After removal of unbound IgE, cells were treated with RN486 for 1 h, and then cross-linked with 1 μg/mI NP 30-bovine serum albumin (Biosearch Technologies, Novato, CA) for 30 min at 37°C. A23187 (calcium ion) (Sigma, St. Louis, MO) was used as a positive control for degranulation. Supernatants were collected and assayed for histamine release by using enzyme immunoassay (Oxford Biomedical Research, Oxford, MI). The percentage of histamine release was obtained by comparing various treatments with the positive control (A23187, 100%).

BioMAP Profiling. To determine the profile of RN486 in more complex primary human cell systems, we had the compound tested at 0.01, 0.1, 1, and 10 μM in the BioMAP Systems platform (BioSeek LLC, South San Francisco, CA) across 12 BioMAP Systems containing early passage primary human cells cultured alone or as cocultures and stimulated with various proinflammatory or immunomodulatory stimuli. These systems have been described previously (Berg et al., 2010) and include (primary human cell types/stimuli): 3C (venular endothelial cells (HuVEC/IL-1β, TNFα, and IFNγ), 4H (HuVEC/IL-4 and histamine), LPS (PBMC and HuVEC/LPS), SAg (PBMC and HuVEC/TCR ligands), BT (B cells and PBMC/anti-IgM and low levels of TCR ligands), BESC (bronchial epithelial cells/IL-1β, TNFα, and IFNγ), BF4T (bronchial epithelial cells and human dermal fibroblasts/TNFα and IL-4), HDF3CGF (human dermal fibroblasts/IL-1β, TNFα, IFNγ, epidermal growth factor, basic fibroblast growth factor, and platelet-derived growth factor-BB), KF3CT (keratinocytes and dermal fibroblasts/IL-1β, TNFα, and IFNγ), CASM3C (coronary artery smooth muscle cells/IL-1β, TNFα, and IFNγ), MyoF (lung fibroblasts/TNFα and transforming growth factor β), and Mphp (HuVEC and macrophages/TLR2). A BioMAP activity profile was generated based on the effects of test compound on the levels of various readout parameters including cytokines or growth factors, expression of surface molecules, and cell proliferation. For more technical details, see Supplemental Table 1.

Experimental Animals

All in vivo procedures were approved by Hoffmann-La Roche’s Institutional Animal Care and Use Committee and performed according to guidelines established by the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996), the Association for the Assessment and Accreditation of Laboratory Animal Care International, and American Veterinary Medical Association Guidelines on Euthanasia (2007). In vivo procedures conducted at Ricerca Biosciences, LLC (Taipei, Taiwan) were approved by its Institutional Animal Care and Use Committee and performed according to the same guidelines as those followed at Hoffmann-La Roche. Animals used for the present studies were male C57BL/6 and female BALB/c mice from The Jackson Laboratory (Bar Harbor, ME), female Lewis rats from Charles River Laboratories, Inc. (Wilmington, MA), and male Wistar rats and female Sprague-Dawley rats from BioLasco, Taipei, Taiwan (under Charles River Laboratories Technology Licensee). RN486 was synthesized at the Chemistry Department at Hoffmann-La Roche in Nutley, NJ. All test compounds were dissolved in vehicle containing 0.5% hypromellose USP, 0.4% polysorbate 80 NF, and 0.9% benzyl alcohol in sterile water USP with pH adjusted to 3.5 ± 0.4.

In Vitro Assay for CD69 in Mouse Splenic B Cells

Splenocytes were isolated from C57BL/6 mice and incubated with RN486 in a round-bottom 96-well plate for 30 min before stimulation with 30 μg/ml anti-mouse IgD antibody (αIgD, LO-MD-6; Accurate Chemical & Scientific, Westbury, NY) for 4 h at 37°C. Immediately after αIgD stimulation, cells were washed in complete RPMI medium and resuspended in BD Biosciences FACs staining buffer at a concentration of 1 × 106 cells/100 μl. Next, cells were incubated with mouse Fc block (BD Biosciences) on ice alone for 10 min and then with fluorescein-conjugated anti-mouse antibodies to CD69 (H1.2F3) and B220 (RA3-6B2), or corresponding isotype controls (BD Biosciences), for 20 min. Labeled cells were then washed to remove excess fluorescein-conjugated antibodies and resuspended in BD Biosciences FACs buffer. Finally, fluorescence signals for CD69 on B220-positive B cells were acquired by using an LSR II flow cytometer (BD Biosciences).
In Vitro and Ex Vivo Assays for B Cell CD69 in Mouse Blood

Fresh whole blood was collected from mice in heparinized tubes and stimulated with αIgD (30 µg/ml) for 4 h at 37°C in a round-bottom 96-well plate. Next, the sample was incubated with a BD Biosciences FACS lysis buffer for 10 min at room temperature to remove red blood cells. Remaining cells were washed, resuspended in BD Biosciences FACS buffer, and incubated with Flc block for 10 min before being incubated with fluorochrome-conjugated antibodies for both CD69 and B220 in the dark for 30 min. Cells were then washed and resuspended in FACS buffer. Fluorescent signals for CD69 on B220+ TCRβ+ cells were acquired and analyzed in the lymphocyte gate by using FlowJo (Treestar). CD69 expression in B220+ cells was reported as the percentage of CD69-positive/total B220+ cells (B220+ CD69+/B220+).

The ability of RN486 to inhibit CD69 expression was examined under both in vitro and ex vivo conditions. For in vitro studies, the compound was incubated with blood for 30 min before αIgD stimulation, whereas for ex vivo experiments, the compound was administered to CIA mice as described in the section below, and blood was collected 2, 6, and 24 h postdose for αIgD stimulation.

Collagen-Induced Arthritis in Mice

DBA1/J male mice, 7 to 8 weeks of age, were fed a breeder diet (mouse chow; Purina, St. Louis, MO). After 1 week of acclimatization, mice received two immunizations with bovine type II antigen (100 µg each) via intradermal injection at their tail bases, first in 0.1 ml of complete Freund's adjuvant (200 µg of Mycobacterium tuberculosis in mineral oil), and 21 days later in 0.1 ml of incomplete Freund's adjuvant (neat mineral oil), for induction of arthritis. Test compound was administered orally once a day for 14 days either preventively starting the day after the injection of incomplete Freund's adjuvant or therapeutically starting whenever their clinical scores reached approximately 4 (therapeutic enrollment treatment). Clinical scores, an index of arthritis, were assessed by using the following criteria: 0, normal with no swelling or redness; 1, swelling and/or redness of paw or one digit; 2, swelling in two or more joints; 3, gross swelling of paw with more than two joints involved; and 4, severe arthritis of entire paw and digits.

At the end of the study, blood was collected for ex vivo CD69 assay as described in the previous section and for anti-type II collagen enzyme-linked immunosorbent assay with a kit from Chondrex Inc. (Richmond, WA). Paws were collected for histopathological analysis.

Collagen Antibody-Induced Arthritis

CAIA was induced in female BALB/c mice by using an arthritogenic kit (Chondrex Inc.). In brief, mice were injected with an antitype II collagen antibody cocktail (four clones; 4 mg/mouse i.p.) on day 0 and LPS (50 µg/mouse) 3 days later. Arthritis scores were assessed by using the same criteria as those used for grading CIA.

Passive Cutaneous Anaphylaxis

Male Wistar rats (five per group), weighing 140 ± 10 g, were passively sensitized with a reaginic (IgE) anti-ovalbumin (anti-OVA) serum (0.05 ml/site i.d.) at two sites on the dorsal back. As a control, one group of rats received saline instead. Animals were challenged 17 h later with OVA (1 mg per rat), which was injected intravenously along with Evans blue dye (5 mg per rat). Animals were sacrificed 30 min later, and skin was photographed and collected for assessing plasma extravasation. Skin tissues surrounding cutaneous wheels (8 mm in diameter) were biopsied by using a skin puncher. Concentrations of Evans blue, a measure of plasma extravasation, were measured at 610 nm in tissue extracts after punch tissues were incubated overnight in 2 ml of formamide at 80°C.

For assessing the effect of RN486, the compound or vehicle was administered 3.5 h before OVA challenge. The effect of the compound, presented as the percentage of inhibition, was determined by comparing OD_{610} in vehicle- and compound-treated animals by using the following formula: 100 - [drug treatment - (-IgE)]/[vehicle control - (-IgE)] × 100.

Reverse Passive Arthus Reaction

Young female Sprague-Dawley rats (five per group), weighing ~125 to 130 g, were fasted overnight before the study. The animals were administered intravenously with saline containing 1% OVA (10 mg/kg) and 1% Evans blue dye 10 min before being injected intradermally with a rabbit anti-OVA IgG (50 µg/25 µl) on the left side of the back at three adjacent locations. As an isotype control, a normal rabbit IgG (50 µg/25 µl) was injected at three locations on the opposite side. As naive controls (-IgG), five rats received injection of saline on both sides of their backs. Vehicle or test compound was administered orally 60 min before the injection of anti-OVA IgG. Four hours after the injection, animals were euthanized with CO2 asphyxiation for collecting skin tissues. Plasma extravasation, an index of inflammation, was determined by measuring dye extravasation in punch biopsies circumventing the injection sites (8 mm) as described in the previous section for PCA, and calculated as a difference between tissues injected with specific anti-OVA and control IgG. The percentage of inhibition was calculated by using the following formula: 100 - [drug treatment - (-IgG)]/[vehicle control - (-IgG)] × 100.

Adjuvant-Induced Arthritis

Complete Freund’s adjuvant, a 0.5-mg M. butyricum (Difco, Detroit, MI) suspension in 100 µl of mineral oil, was injected intradermally into the tail base of rats to induce arthritis. Paw swelling, an index of arthritis, was quantified by measuring the volume of hind paws by using water displacement plethysmography on days 0, 7, 14, and 18. For assessing the ability of RN486 to inhibit AIA, the compound was administered to animals between days 7 and 18. As a control, vehicle was administered to two groups of rats, one with AIA and the other without. At the end of the study, spleen, hind paws, and serum were collected for measurement of splenomegaly, histopathological analysis, and profiling of serum proteins. Profiling of serum proteins was performed by using RRM RodentMAP Antigen 2 at Rules Based Medicine (Austin, TX). Samples from naive, vehicle, and 30 mg/kg RN486 groups in RN486 monotherapy and naive, vehicle, RN486 (10 mg/kg), MTX (30 mg/kg), and RN486 + MTX groups in combination therapy were included in the RRM profiling experiment and analyzed for assessing the effects of monotherapy and combination therapy with RN486 and MTX on serum inflammatory markers.

Histopathological Analysis

Hind paws from CIA mice or rats were collected into 10% neutral buffered formalin. After decalcification in 10% formic acid for 5 days, paws from AIA and CIA mice were embedded in paraffin and sectioned at 8 µm. Paws from rats were sectioned in the sagittal plane, whereas those from mice were in the frontal plane. Tissue sections were then stained with toluidine blue. Inflammation (infiltration of inflammatory cells), pannus, cartilage damage, and bone resorption were scored in a double-blinded fashion by a board-certified pathologist at BoulderPath, Inc. (Boulder, CO) using standard criteria, with 0 being normal and 5 being the most severe. For AIA rats, pannus and cartilage damage are not reported because these changes were not prominent in the model.

Micro Computer Tomography Imaging

MicroCT imaging was performed by an imaging specialist at Covance, Inc. (Greenfield, IN) on samples from selected groups (vehicle and 3 and 100 mg/kg RN486) of animals in the CIA study before the samples were used for histological analysis. Data were acquired at 28-µm isotropic voxel size, photon energy of 30 keV, and current of 80 µA. Exposure time per frame was 1700 ms, and the total imaging...
time per sample was approximately 70 min. Three-dimensional image rendering and calculation of bone mineral density were performed by using Microview (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

### Statistical Analysis

Standard nonlinear regression analysis was performed by using custom Excel templates to determine concentration response curves and IC$_{50}$ values in vitro assays. The IC$_{50}$ values reported are averages from at least three studies conducted in duplicate or triplicate. For in vivo studies, one- and two-factor comparisons were performed, respectively, by using one-way analysis of variance plus Dunnett's post-test and two-way analysis of variance plus Bonferroni's post-test. All statistical analysis for in vivo studies was performed by using Prism 5.01 (GraphPad Software, Inc., San Diego, CA).

### Results

**Potency, Selectivity, and Pharmacokinetics of RN486.** RN486 was selected from a large number of proprietary Btk inhibitors based on its potency, selectivity, and pharmacokinetic profile. In the enzymatic assay, the compound potently inhibited Btk kinase activity with an IC$_{50}$ of 4.0 nM. It binds the enzyme in a competitive manner as demonstrated in a time-resolved FRET-based competitive binding assay with an IC$_{50}$ of 0.3 nM. RN486 was shown to be highly selective when tested against a panel of 369 kinases in the Kinomescan. In the assay, the compound exhibited a strong and competitive binding to Btk with a $K_d$ of 0.31 nM and a high degree of selectivity over almost all other kinases, including Syk and Janus kinase (JAK).

The enzyme that was most potently inhibited next to Btk was Ste20-like kinase (SLK), for which the compound showed a 139-fold selectivity (Table 1). When tested in the rat and mouse, RN486 exhibited an excellent pharmacokinetic profile. In the rat, it reached the maximal concentration of 2.5 μM at 4.5 h when dosed orally at 20 mg/kg and showed a half-life of 9.8 h in the blood when administered intravenously. In the mouse, the compound reached the maximal concentration of 6.0 μM at 3 h and a trough concentration of 1.0 μM at 24 h when dosed orally at 30 mg/kg.

**RN486 Blocks both BCR and FcR Signaling.** To determine the impact of Btk inhibition on BCR signaling, we examined the effects of RN486 on several key events in the BCR signaling cascade ranging from phosphorylation of Btk and PLCγ2 to calcium influx and CD69 expression in B cells with isolated human B cells, Ramos B cells, or whole blood. Stimulation with αIgM induced phosphorylation of Btk (Tyr551) and PLCγ2 (Tyr1217) in isolated B cells, calcium influx in Ramos cells, and CD69 expression in blood CD20 + B cells, whereas pretreatment of these cells with RN486 markedly inhibited all these signaling events, reducing both phospho-Btk and phospho-PLCγ2 to below the baseline level without affecting the levels of total Btk or PLCγ2 (Fig. 1) and blocking calcium influx (Fig. 2A) and CD69 expression in a concentration-dependent manner, with respective IC$_{50}$ values of 25.9 and 21.0 nM (Fig. 2B) (Table 2).

We also evaluated the ability of RN486 to block FcR-mediated responses by examining its effects on IgG-coated bead-induced TNFα release in monocytes and anti-NP IgE- and antigen-induced histamine release in mast cells. These responses are mediated by FcγR (Debets et al., 1988) and FcεRI (Ishizaka et al., 1983), respectively. The addition of IgG beads to monocytes induced a significant increase in TNFα production. RN486 inhibited TNFα production in a concentration-dependent manner (Fig. 2C), with an IC$_{50}$ of 7.0 nM (Table 2). The compound displayed a similar inhibitory effect on FcεR-mediated mast cell degranulation, reducing IgE- and antigen-mediated release of histamine to the baseline level, with an average IC$_{50}$ value of 2.9 nM (Fig. 2D; Table 2).

To examine whether RN486 was also active in mice, we assessed the effect of the compound on BCR-mediated CD69 expression in mouse splenocytes and whole blood. As in human B cell-based assays, the compound potently inhibited αIgD-stimulated CD69 expression in a concentration-dependent manner in both splenic (Fig. 2, E and F) and blood B cells (Fig. 2G), with a IC$_{50}$ of 2.1 ± 0.9 and 4.2 ± 3.2 nM, respectively (Table 2).

**RN486 Displays a Selective B Cell Inhibitory Profile in BioMAP Systems.** Having demonstrated the ability of RN486 to potently block both BCR- and FcεR-mediated biological responses in the tailored cellular assays for immunoreceptor signaling, we tested the compound in a panel of more complex primary human cell systems, namely the BioMAP Systems, that would permit us to not only further define the mechanisms of action of the compound, but also study its potential for causing off-target effects (Berg et al., 2010). The BioMAP Systems panel consists of 12 primary human monoculture or cocultures stimulated with various proinflammatory or immunomodulatory stimuli, including PBMC + endothelial cell cocultures stimulated with LPS (LPS System), or superantigen (cocktail of ligands for T cell receptor) (SAg System), and PBMC + B cell coculture stimulated with anti-IgM and mild TCR ligands (BT System). When tested at 0.01, 0.1, 1, and 10 μM, RN486 inhibited the proliferation of B cells and the production of IgG and cytokines (IL-2 and IL-6) in a concentration-dependent manner.
in the BT system without significantly affecting various read-out parameters in 11 other BioMAP Systems, including the SAg and LPS systems (Fig. 3).

**RN486 Blocks Type I and Type III Hypersensitivity Responses in Rats.** Data from cellular assays demonstrate that RN486 blocks both FcεR- and FcγR-mediated biological responses and suggest that the compound may be effective at blocking immune hypersensitivity responses mediated by these receptors in vivo. To test this hypothesis, we assessed RN486 in rat models of PCA and rPAR. In the PCA model, a type I cutaneous hypersensitivity response was induced by anti-OVA IgE (IgE intravenously)
and a subsequent challenge with the antigen OVA via intradermal injection. OVA challenge caused a severe cutaneous hypersensitivity response as demonstrated by the appearance of skin wheals and dye extravasation around the sites of injection in IgE-sensitized but not nonsensitized rats (Fig. 4A, top left, nonsensitized; top center, sensitized). RN486 markedly inhibited the response, as demonstrated by the diminished wheal reaction (Fig. 4A, top right, RN486 30 mg/kg) and a dose-dependent inhibition of plasma extravasation (Fig. 4B). The maximal effect obtained was 91% (100 mg/kg), more than the 69% inhibition noted with cyproheptadine, a nonselective antihistamine agent used as a positive control (Fig. 4B).

In the rPAR model, a type III cutaneous hypersensitivity response was induced in reverse order to PCA by first injecting the antigen OVA (intravenously) and then anti-OVA IgG (intradermally). Anti-OVA IgG injection produced a robust inflammatory response around the site of the injection, resulting in a significant increase in plasma extravasation (Fig. 4A, top left, controls; bottom center, vehicle treatment). Dexamethasone, a corticosteroid anti-inflammatory drug, significantly inhibited the response by 50% (Fig. 4A, bottom left, controls; bottom center, vehicle treatment). Dexamethasone, a corticosteroid anti-inflammatory drug, significantly inhibited the response by 50% (Fig. 4A, bottom left, controls; bottom center, vehicle treatment). Dexamethasone, a corticosteroid anti-inflammatory drug, significantly inhibited the response by 50% (Fig. 4A, bottom left, controls; bottom center, vehicle treatment).

RN486 Displays Efficacy on Immune Arthritis Induced by both Active and Passive Immunization in Mice. To determine the effect of RN486 on the development of immune arthritis, we tested the compound in two murine models of RA (CIA and CAIA). In the CIA model, we first tested the compound in a preventive mode. When administered orally at doses of 3, 30, and 100 mg/kg q.d. for 14 days starting 1 day after the second immunization, RN486 inhibited arthritis as measured by clinical scores in a dose-dependent manner, with a complete inhibition at 100 mg/kg, similar to dexamethasone (Fig. 5A). When examined ex vivo, the compound almost completely inhibited IgD-stimulated CD69 expression in blood B220+ cells at 3 and 6 h postdose at all doses and by approximately 50 to 80% 24 h postdose. Under the same conditions, the compound was without effect on LPS-induced CD69 expression (Fig. 5B). RN486 also attenuated the production of anti-type II collagen antibody in a dose-dependent manner, by as much as 85% at 100 mg/kg, which is more than the 60% inhibition by dexamethasone (Fig. 5C). The inhibitory effect on the antibody production correlates highly with that on CD69 expression noted at 24 h ($r^2 = 0.77$). Histologically, RN486 produced a dose-dependent inhibitory effect on inflammation, pannus formation, cartilage damage, and bone resorption (Fig. 5D). The bone-protective effect was also confirmed by microCT analysis, which revealed a marked improvement in bone density in RN486-treated mice over vehicle-treated animals that displayed severe and widespread bone loss (Fig. 5E, left, vehicle; middle and right, RN486 3 and 100 mg/kg, and F, bone mineral density of tarsal bone of hind paws).
Next, we tested RN486 in mice with established arthritis in a therapeutic dosing regimen. In the study, individual mice were randomly enrolled into either the vehicle- or compound-treated groups when their clinical scores reached 3 to 5 and then treated with RN486 (30 mg/kg p.o. q.d.) consecutively for 14 days. Mice treated with vehicle displayed further increases in their clinical scores (Fig. 6A). By comparison, the progression of arthritis was completely blocked in animals treated with RN486 as demonstrated by clinical scores (Fig. 6A) and supported by histopathological analysis (Fig. 6B; Supplemental Fig. 1). In contrast to preventive treatment, therapeutic dosing of RN486 resulted in only a modest and nonsignificant reduction in the titer of anti-type II collagen antibodies (Fig. 6C), suggesting that selective Btk inhibition attenuates arthritis development by blocking downstream effector function mediated by arthritogenic anti-type II collagen antibodies. To validate this hypothesis, we tested RN486 in the CAIA model, in which arthritis was induced by exogenous anti-type II collagen antibodies. In support of the hypothesis, the compound completely prevented anticollagen antibody-induced arthritis (Fig. 6D).

RN486 Inhibits Inflammation and Bone Erosions in Adjuvant-Induced Arthritis Either Alone or in Combination with Methotrexate. To further validate the anti-rheumatic potential of RN486, we tested the compound in rat AIA, a standard RA model for testing small-molecule anti-rheumatic and anti-inflammatory drugs such as low-dose methotrexate (Welles et al., 1985) and CP-690,550 (tofacitinib), a new JAK inhibitor (Milici et al., 2008). When administered alone, RN486 displayed robust and dose-dependent inhibitory effects on both joint and systemic inflammation or immune response as measured by paw swelling (Fig. 7A) and splenomegaly (Fig. 7B). A maximal ~80% inhibition on paw swelling was observed at 30 mg/kg. The effect was associated with a significant suppression of histopathological changes of both inflammation and bone erosions (Fig. 7, C and D; Supplemental Fig. 2). It is noteworthy that the concentration response relationship in the model correlates with that noted in the PCA model (Fig. 7E).

Combination therapy with low-dose methotrexate is an important treatment option for patients with RA. We therefore determined the potential of RN486 for combined therapy with methotrexate. To identify a suboptimal or low dose of methotrexate for combination study, we tested methotrexate alone in the AIA model at doses ranging from 0.025 to 0.25 mg/kg. Methotrexate displayed a dose-dependent inhibitory effect on both paw inflammation and splenomegaly in AIA rats, attenuating paw swelling by ~50 and 100%, respectively, at 0.075 and ~0.15 mg/kg (Fig. 8A). We then tested RN486 and methotrexate at their respective suboptimal doses, 10 and 0.075 mg/kg, alone or in combination in the AIA model to assess the combined effect. As in the monotherapy studies, both RN486 and methotrexate attenuated paw swelling by approximately 50% when tested alone at the suboptimal doses. When combined, the two compounds completely eradicated paw swelling (Fig. 8B), splenomegaly (Fig. 8C), and histopathological changes of inflammation and bone erosions (Fig. 8, D and E).

RN486 Reduces Blood Inflammatory Markers in AIA. RA is a systemic autoimmune disease that causes inflammation not only in the joint but also systemically as demonstrated by increased levels of acute-phase response proteins and other inflammatory markers in the blood. Consequently, effective treatment of RA by using antirheumatic drugs in patients is frequently associated with significant reductions in serum inflammatory markers that are indicative of systemic inflammation. To investigate whether Btk inhibition also modulates serum inflammatory markers, we analyzed 59 serum protein...
Fig. 5. Anti-inflammatory and disease-modifying effects of RN486 in the mouse CIA model. A, dose-dependent attenuation of clinical scores by RN486 (n = 14/group). B, inhibition of αIgD, but not, LPS-induced CD69 expression in ex vivo whole-blood assay at 24 h postdose. CD69 expression in B220⁺ cells was induced ex vivo with αIgD or LPS in terminal whole blood from subgroups of mice 24 h postdose. CD69 expression in nonstimulated (~αIgD) was also examined and used as a baseline control (n = 5/group). C, attenuation of plasma levels of anti-type II collagen antibody (total IgG) by RN486 and dexamethasone. D, histopathological analysis of inflammation, pannus formation, cartilage damage, and bone resorption in the hind paws of a subgroup of mice (n = 7/group). E, representative microCT images from a vehicle-treated mouse (left) or RN486-treated mouse (center, 3 mg/kg; right, 100 mg/kg) depicting the bone-protective effect of RN486. F, quantitative analysis of bone mineral density (BMD) in tarsal bone of hind paws from a region of interest (3.2 × 1.2 × 2.2 mm) in the microCT images (n = 7/group); *, p < 0.05 and **, p < 0.01 compared with vehicle-treated group.

Fig. 6. Inhibitory effect of RN486 on the effector phase of immune arthritis in mice. A to C, inhibitory effects in the CIA model on clinical scores (A), histopathological changes in inflammation, pannus, cartilage damage, and bone resorption (B) and plasma levels of anti-type II collagen Ab (C). n = 12/group for vehicle and 14/group for RN486. **, p < 0.01 versus vehicle. D, inhibitory effect on clinical scores in the CAIA model. n = 6 for vehicle; 8/group for RN486.
analytes by using the RBM RodentMap panel in serum samples from both monotherapy and combotherapy studies with RN486. Among the 59 analytes, 23 were below the limit of detection (Supplemental Table 2). Of those detected (36), 10 were significantly increased in vehicle-treated AIA rats compared with nonarthritic rats (Supplemental Table 2), four of which, serum C reactive protein (CRP), haptoglobin, vascular cell adhesion molecule and chemokine ligand 5 (regulated on activation normal T cell expressed and secreted), were significantly attenuated by both combotherapy and monotherapy with RN486 (Fig. 9, A–D). Serum levels of these inflammatory markers correlated strongly with paw swelling as revealed by correlation analysis between paw volume and serum CRP ($R^2 = 0.6$; Fig. 9E). Among the remaining six, five analytes, interferon-inducible protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage colony stimulating factor, macrophage inflammatory protein-2, and tissue inhibitor of metalloprotease-1, were significantly ($p < 0.05$) reduced by combotherapy.

**Discussion**

Mutation of Btk in both humans and mice results in B cell immunodeficiency, implicating the enzyme as a key regulator of humoral immunity and a potential target for developing drugs to treat RA and other autoimmune diseases. Concordantly, we have demonstrated that a novel Btk inhibitor, RN486, blocks both BCR- and FcR-mediated hypersensitivity responses and mitigates the development of immune arthritis in rodent models of RA.

RN486 is a reversible Btk inhibitor from a structural class distinct from previous Btk inhibitors, 1-[(3R)-3-[4-amino-3-(4-phenoxypyphenyl)pyrazolo[3,4-d]pyrimidin-1-yl]piperidin-1-yl]prop-2-en-1-one (PCI-32765) (Honigberg et al., 2010), N-[3-[4,5-dihydro-4-methyl-6-[4-(4-morpholinylcarbonyl)phenyl]amino]-5-oxo-2-pyrazinyl]-2-methylphenyl]-4-(tert-butyl)benzamide (CGI1746) (Di Paolo et al., 2011), and R-N-(3-(6-(4-(1,4-dimethyl-3-oxopiperazin-2-yl)phenylamino)-4-methyl-5-oxo-2-pyrazinyl)-2-methylphenyl)-4,5,6,7-tetrahydrobenzo[b]thiophene-2-carboxamide (GDC-0834) (Liu et al., 2011). In contrast to the irreversible covalent binding PCI-32765, RN486 and the two previous Btk inhibitors, CGI1746 and GDC-0834, are reversible Btk inhibitors, which are desirable for their further development as therapeutics. RN486 has an excellent potency and selectivity profile. Specifically, RN486 exhibited a subnanomolar potency for Btk and a high de-
degree of selectivity over a large panel of 396 kinases, including Syk and JAK, two validated RA targets (Kremer et al., 2009; Weinblatt et al., 2010). The enzyme that was inhibited the most by RN486 next to Btk was SLK, an enzyme not implicated in autoimmune responses, with a 139-fold selectivity. Together, these data indicate that RN486 is a selective and potent inhibitor for Btk.

RN486 is able to block the signaling of BCR as demonstrated by a marked inhibition of phosphorylation of both Btk and PLCγ2 in B cells, similarly to previous Btk inhibitors (Honigberg et al., 2010; Di Paolo et al., 2011). It is noteworthy that the effect was observed in not only αIgM-stimulated, but also nonstimulated, B cells, suggesting that BCR was probably already activated to a certain extent under basal experimental conditions. It is noteworthy that the compound retained potency and selectivity in cell-based assays, strongly inhibiting key events downstream of BCR as evidenced by the blockage of BCR ligand-induced calcium influx in Ramos cells and CD69 expression in whole blood. Likewise, the compound inhibited FcR-mediated responses in effector immune cells, abrogating mast cell degranulation and TNFα production in monocytes induced, respectively, by ligands that have been shown to specifically activate FcγRI and FcγR (Trotta et al., 1996; Okayama et al., 2001). RN486 is also active in mice as demonstrated by its inhibition of BCR-mediated CD69 expression in both splenic and blood B cells. In contrast, the compound failed to affect LPS-induced CD69 expression in blood, indicating that the Btk inhibitor
only blocks immunoreceptor-mediated responses in immune cells.

The same holds true in more complex BioMAP primary human cell systems. Although still limited in their complexity compared with disease tissues, the BioMAP Systems are designed to model disease environments in vitro by exposing primary human cells, mostly in coculture, to combinations of disease-related stimuli. More importantly, these systems have been extensively studied with a broad range of therapeutics and shown to have the capacity for identifying mechanisms of drug actions and potential toxicities (Berg et al., 2006, 2010). In these assays, RN486 selectively inhibited the function of B cells in a BCR and TCR ligand-stimulated B cell/PBMC coculture without affecting the function of other cell types including T cells in the same culture and macrophages stimulated with LPS. The evidence points to a specific mechanism-based inhibition of B cell activation by BCR stimulation. Also importantly, the lack of effects on other cell types was consistent with the high degree of selectivity of the compound and indicative of a low potential for RN486 to elicit off-target effects.

The ability of RN486 to block immunoreceptor-mediated responses was further demonstrated in vivo in rat models of PCA and rPAR, in which the compound blocked both type I and III hypersensitivity responses, known to be induced by FcεR-dependent mast cell degranulation, and FcγR-dependent activation of neutrophils and other innate immune cells, respectively (Sakurai et al., 2004; Ellsworth et al., 2008). In both models, RN486 was superior to the benchmark compound cyproheptadine or dexamethasone, indicating that se-
lective disruption of FcR signaling by inhibiting Btk is more effective than blocking distal events such as inhibiting the activity or generation of inflammatory mediators with the benchmark compounds. Together, these data unequivocally demonstrate that, as in human cellular systems, Btk plays an essential role in mediating FcR-mediated responses in animal models that faithfully mimic human hypersensitivity conditions.

The same holds true in mouse models of RA, in which RN486 prevented not only the production of anti-type II collagen, but also the development of CAIA, which is mediated by FcR (Kagari et al., 2003). At the doses (3–100 mg/kg) that produced efficacy in the CIA model, RN486 nearly completely inhibited ex vivo BCR ligand-induced CD69 expression for the first 6 h and by 50 to 80% at 24 h postdose. These findings are consistent with a previous report demonstrating that an approximate threshold of 60% pBTK inhibition is required for antiarthritic activities (Liu et al., 2011). It is noteworthy that CD69 inhibition at the 24-h time point correlates well with the suppression of anti-type II collagen antibody. These data suggest that a selective Btk inhibitor may produce efficacy by blocking both the production and effector function of arthritogenic antibodies that underlie the initiation and propagation of autoimmune arthritis through a selective inhibition of BCR and FcR signaling. Together, our findings in the mouse RA models not only confirm previous evidence for the inhibition of immune arthritis by both genetic mutation and pharmacological inhibition of Btk (Honigberg et al., 2010; Di Paolo et al., 2011), but also provide additional mechanistic insights through the demonstration of a strong correlation between the inhibition of arthritogenic antibodies and the suppression of BCR-dependent CD69 expression.

In addition to abrogating immune arthritis in mouse CIA, which highly depends on B cells and humoral autoimmunity, RN486 prevented the development of AIA, a model that is driven mainly by T cells and innate immune responses (Spargo et al., 2006). In the model, RN486 produced not only a robust efficacy on joint inflammation and splenomegaly when dosed alone, but also a strong additive effect when combined with a low dose of methotrexate. It is noteworthy that RN486 exhibited a similar concentration response relationship in the model to that in PCA, suggesting that the concentrations required for blocking an immunoreceptor-dependent response are sufficient for efficacy. It is not clear whether BCR or FcR is involved because B cell and humoral autoimmunity have not been shown to contribute to AIA. Other possible receptors include CSF1R and myeloid DAP12-associating lectin-1, both of which depend on ITAM-containing DAP12 for signaling, and are implicated in immune arthritis in rodents (Huang et al., 2009; Joyce-Shaikh et al., 2010). Although the exact mechanism remains to be defined, our data suggest that Btk inhibitors may also produce efficacy in autoimmune conditions in which T cell and innate immune responses dominate. It is noteworthy that evidence of an additive effect between RN486 and low-dose methotrexate provides a preclinical proof of principle for potential combotherapy.

In both monotherapy- and combotherapy, RN486 strongly protects bone in arthritic joints against erosions, indicative of disease-modifying activity. Although the effect may be secondary to diminished inflammation, a Btk inhibitor may protect bone directly by inhibiting osteoclast differentiation. This is because osteoclast differentiation in both RA models and patients is mediated by RANKL (Stolina et al., 2005; Leibbrandt and Penninger, 2009), which requires both FcγR and CSF1R/DAP12, consequently Btk, for the action. Indeed, Btk deficiency impairs differentiation of osteoclasts from bone marrow cells stimulated with RANKL (Lee et al., 2008). Additional studies are needed for assessing the ability of RN486 to modulate RANKL-induced osteoclast differentiation and bone resorption.

In addition to inhibiting inflammation and bone erosions, treatment with RN486 either alone or in combination significantly attenuated the levels of several serum proteins, including CRP, vascular cell adhesion molecule 1, and regulated on activation normal T cell expressed and secreted, in the AIA model. It is noteworthy that these proteins are also reduced in patients with RA in response to effective antirheumatic therapies (Klimiuk et al., 2007; Yang et al., 2009). Reduction in CRP, as well as other serum proteins, in AIA correlates with improvement in paw swelling, suggesting that the proteins may be useful markers for monitoring responses to RN486. Several other proteins such as interferon inducible protein 10, MCP-1, MCP-3, macrophage inflammatory protein-2, tissue inhibitor of metalloprotease 1, and vascular endothelial growth factor, on the other hand, are reduced only by combotherapy and may be used to measure treatment responses to both RN486 and methotrexate.

In summary, we have demonstrated that a selective Btk inhibitor, RN486, blocked BCR- and FcR-mediated biological and immune responses in both human cellular assays and rodent models, providing evidence for mechanism-based actions relevant to human diseases. More importantly, we demonstrated that RN486 produced a robust efficacy in two standard rodent models of RA at concentrations that effectively block immunoreceptor-mediated pharmacodynamic responses, i.e., CD69 in mice and PCA in rats. Together, our data show that Btk is an indispensable regulator of immunoreceptor-mediated responses in both rodent and humans. Because these immunoreceptor-mediated responses are conserved between rodents and humans, and are essential for the development of immune arthritis in both species, our findings may be clinically relevant and support the development of selective Btk inhibitors as RA therapeutics.

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References
Btk Inhibitors for Rheumatoid Arthritis


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