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SB1578, a Novel Inhibitor of JAK2, FLT3, and c-Fms for the Treatment of Rheumatoid Arthritis

Babita Madan,1 Kee Chuan Goh,1 Stefan Hart, Anthony D. William, Ramesh Jayaraman, Kantharaj Ethirajulu, Brian W. Dymock, and Jeanette M. Wood

SB1578 is a novel, orally bioavailable JAK2 inhibitor with specificity for JAK2 within the JAK family and also potent activity against FLT3 and c-Fms. These three tyrosine kinases play a pivotal role in activation of pathways that underlie the pathogenesis of rheumatoid arthritis. SB1578 blocks the activation of these kinases and their downstream signaling in pertinent cells, leading to inhibition of pathological cellular responses. The biochemical and cellular activities of SB1578 translate into its high efficacy in two rodent models of arthritis. SB1578 not only prevents the onset of arthritis but is also potent in treating established disease in collagen-induced arthritis mice with beneficial effects on histopathological parameters of bone resorption and cartilage damage. SB1578 abrogates the inflammatory response and prevents the infiltration of macrophages and neutrophils into affected joints. It also leads to inhibition of Ag-presenting dendritic cells and inhibits the autoimmune component of the disease. In summary, SB1578 has a unique kinase spectrum, and its pharmacological profile provides a strong rationale for the ongoing clinical development in autoimmune diseases. The Journal of Immunology, 2012, 189: 4123–4134.

Rheumatoid arthritis (RA) is a debilitating and systemic autoimmune disease affecting ~1% of the world population (1). The disease is characterized by chronic inflammation of synovial joints leading to the destruction of articular cartilage and erosion of the bone (2). The understanding of the complex cause of RA has considerably improved. Studies have established that the early phase of this autoimmune disease development involves Ag presentation leading to the production of autoreactive Th cells. This is followed by an excessive recruitment and retention of inflammatory cells, primarily lymphocytes, neutrophils, and macrophages in the synovium, which leads to pannus formation (3, 4). Dysfunctional Th cell responses lead to the skewing of cytokine repertoire produced by Th cell subsets causing an overproduction of proinflammatory cytokines exemplified by IL-1β, IL-2, IL-6, IL-12, IL-17, and IL-23 at the expense of anti-inflammatory cytokine production (5). Consequently, the RA synovium is characterized by elevated levels of proinflammatory cytokines and chemokines, including TNF-α, IL-1β, IL-6, and KC/GRO (6, 7).

Multiple strategies targeted at the neutralization of proinflammatory cytokines using mAbs (e.g., anti–TNF-α, anti–IL-6R), soluble receptors (e.g., TNFR-Fc), or antagonists (e.g., IL-1Ra) have been shown to be efficacious for treatment of arthritis in some patients (8–12). However, considerable adverse effects such as increased risk of microbial infections, development of secondary diseases, and other drawbacks such as primary or secondary unresponsiveness remain (13). These drawbacks have encouraged efforts to develop alternative modalities based on targeting of key cytokines involved in the pathogenesis of RA. Emerging evidence suggests that small-molecule inhibitors of protein kinases involved in cellular signaling of cytokines may be viable candidates for novel RA therapies (14, 15).

The JAKs, a family of cytoplasmic tyrosine kinases comprising JAK1, JAK2, JAK3, and TYK2, mediate signaling by association with type I and type II cytokine receptors. JAK activation leads to the phosphorylation of downstream substrates, the STAT proteins, with type I and type II cytokine receptors. JAK activation leads to their nuclear translocation and activation of target genes (16). Dysfunctional JAK–STAT signaling has been implicated in various hematological malignancies and other pathological conditions such as allergies, asthma, and RA (16, 17). As JAKs play an essential role in the cellular signaling pathways involved in regulating the immune system and several important physiological processes such as hematopoiesis, targeting of all the JAK family members simultaneously may lead to immunosuppression and adverse consequences. Therefore, identification of small-molecule inhibitors specifically targeting the JAKs involved in the signal transduction of key cytokines and chemokines associated with the pathogenesis of RA would be beneficial (18, 19). JAK2 plays an essential role in the signaling of proinflammatory cytokines involved in the pathogenesis of RA (20). This suggests that JAK2-specific inhibitors may be differentiated from other JAK inhibitors and produce therapeutic benefits in several inflammatory autoimmune diseases, including RA.

In addition, signaling through c-Fms, a tyrosine kinase receptor, is also increased in several pathologies that involve chronic activation of tissue macrophages, including RA. Elevated levels of its ligand M-CSF are observed in the joints of RA patients, contributing to the development of macrophages and osteoclasts, which are the mediators of bone erosion (21–24). Blockade of the M-CSF–c-Fms axis has been shown to inhibit the progression of arthritis in animal models indicating that it may play a pivotal role in the pathogenesis of RA (25, 26). Signaling via FLT3 is also important for RA pathogenesis as FLT3-mediated signaling is essential for the in vivo differentiation of dendritic cells (DCs).
(27). DCs are the most potent APCs and play an important role in presentation of autoantigens thereby activating self-reactive CD4+ T lymphocytes. In addition, elevated levels of FLT3 ligand (FLT3L) are observed in the synovial fluids of RA patients (28). Moreover, FLT3L can substitute for M-CSF in osteoclast differentiation and function, thereby contributing to the bone erosion in the arthritic joints (29). Hence, small molecules with inhibitory activity against more than one of these target kinases may exhibit an increased potential for treatment of RA.

In this study, we report that SB1578 is a novel, low m.w. compound with activity against JAK2, FLT3, and c-Fls. We show that SB1578 confers significant benefits in two rodent models of RA: the adjuvant-induced arthritis (AIA) in rats and collagen-induced arthritis (CIA) in mice. In both models, SB1578 prevents disease progression and reduces severity along with histological improvement of the joints. Our findings indicate that its efficacy is associated with an abrogation in proinflammatory cytokine production and the concomitant infiltration of inflammatory cells. These results provide a compelling rationale for the clinical evaluation of SB1578 in RA and other inflammatory diseases.

Materials and Methods

Compounds

SB1578, depicted in Supplemental Table I, was synthesized at S*BIO Pte Ltd (Singapore). Stock solutions were prepared from the citrate salt in DMSO, and the final DMSO concentration in all biological assays was 0.1%. All doses used in animal studies are stated as free base equivalent.

In vitro kinase activity assays

JAK1, JAK2, JAK3, Tyk2, FLT3, CDK2, Nek2, and TTK recombinant kinases were purchased from Cell Signaling Technology (Danvers, MA). All assays (except TTK) used the PKLight HTS Protein Kinase Assay Kit from Cambrex Corporation (East Rutherford, NJ). For TTK, FlashPlate assay from PerkinElmer (Waltham, MA) was used. IC50 values were calculated from concentration-response curves using Prism v5.0 software (GraphPad Software, La Jolla, CA). The assays for BTK, ALK, and spleen tyrosine kinase were performed at Millipore (Dundee, U.K.), c-Kit and Arf5 assays were performed at ProQinase (Freiburg, Germany), and AXL, insulin receptor, c-Fms, and RET assays were performed at Reaction Biology Corporation (Malvern, PA).

Animal care

The rat AIA and the murine CIA studies were performed at Bolder BioPATH (Boulder, CO). The studies were approved by the Bolder BioPATH Institutional Animal Care and Use Committee for compliance with regulations. Animals were housed in standard cages and were allowed access to food and water ad libitum. Temperatures ranging between 67 and 76˚F, 30–70% relative humidity, and 12 h of light and dark cycle were maintained.

Rat adjuvant-induced arthritis

Male Lewis rats from Charles River Laboratories (Wilmington, MA) weighing 180–220 g were injected with 60 mg/ml lipoidal amine (Bolder BioPATH) emulsified in 100 μl of CFA (Sigma, St. Louis, MO) at the tail base. SB1578 or methotrexate as a solution in PBS was administered i.p. twice daily for 14 d. Caliper measurements of the ankle joints were made prior to onset of swelling (day 7) and thereafter every day until the end of the study. The data for ankle joint diameter was analyzed by determining the area under the dosing curve (AUC). Body weights for all the animals were also recorded daily. At the completion of the study on day 14, animals were euthanized: hind paws were transected at the level of medial and lateral malleolus and fixed for histopathological analysis. Spleens and livers were also excised and weighed prior to fixation in formalin, and their weights relative to the body weight were calculated.

Collagen-induced arthritis

Male B10/RIII mice, 6–8 wk old, were obtained from The Jackson Laboratory (Bar Harbor, ME). To induce arthritis, the mice were injected at the base of the tail with 300 μg bovine type II collagen (Elastin Products, Owensville, MO) emulsified in CFA (Sigma). After 15 d, the mice were given a booster of 300 μg type II collagen.

For the prophylactic protocol, oral treatment with SB1578 or dexamethasone was initiated on the day of primary immunization (day 0) and continued for the duration of the experiment. Clinical scores for the mice were determined for each of the four paws on study days 16–28. The scores for all the four paws were given using the following criteria: 0, normal joints; 1, swelling in one digit or joint or minimal diffuse erythema; 2, swelling in two digits or joints or mild diffuse erythema; 3, swelling in three digits or joints or moderate diffuse erythema; 4, swelling in four digits or joints or marked diffuse erythema; 5, severe diffuse erythema and severe swelling of the entire paw and rigid joints. The sum of scores for all four paws from each mouse was used as the total clinical score.

For the therapeutic protocol, mice were randomized into treatment groups and oral treatment was initiated only after the onset of arthritis occurred and swelling was established in at least one paw (usually between days 18 and 22). Treatment was continued twice daily for 10 d. Clinical scores for all the four paws were determined thereafter using the same criteria as for the prophylactic protocol. The data for paw scores (means for animals) were analyzed by determining the AUC for arthritis days 16–28. The daily mouse scores were obtained and the AUC between the treatment days and the final day was computed. Means for each group were determined, and percentage inhibition from arthritis controls was calculated by comparing values for treated and normal animals.

Histopathological analysis

Right and left hind paws and spleens of rats and all the four paws and knees of mice were fixed in 10% neutral buffered formalin and processed for staining. Briefly, the paws and knees were decalcified, dehydrated through a graded series of ethanol, and infiltrated with and embedded in paraffin. The paws and spleens of rats were stained with H&E whereas the paws and knees of mice were stained with toluidine blue.

Stained sections of the tissues from all the animals were examined microscopically by a certified veterinary pathologist. H&E-stained arthritic joints of rats were scored on a scale of 0–5 for bone resorption and inflammation. To semiquantitate the inflammatory edema, measurements were taken from the dorsal skin surface (in flexion angle) to ventral skin surface (across the tarsal joints). Inflammation was scored as 0, normal; 1, minimal infiltration of inflammatory cells in the periarticular tissue; 2, mild infiltration; 3, moderate infiltration with moderate edema; 4, marked infiltration with marked edema; 5, severe infiltration with severe edema. The spleens from rats were also scored on a scale of 0–5 based on inflammation, extramedullary hematopoiesis, and lymphoid atrophy.

Histological assessment of arthritis severity of mice was made by blinded evaluation of toluidine blue-stained joint sections. Each of the joints was analyzed, and individual parameters were scored on a scale of 0–5 for increasing degrees of pannus formation, damage to the surface of the articular cartilage, bone resorption, and inflammatory cell infiltrates within the synovial space and periarticular tissue of the affected joints. The sums and means for each of the individual parameters for all the six joints for each animal were determined. Parameters for the various groups were then compared with disease control animals.

Cells

CD4+ T cells isolated from human PBMCs, with >95% purity were obtained from AllCells (Emeryville, CA). The THP-1 cell line was purchased from American Type Culture Collection and maintained as recommended.

Immunoblot analysis

Cells were washed with PBS and then lysed using modified radio-immunoprecipitation buffer [50 mM Tris-HCl, 150 mM NaCl, 1% sodium deoxycholate, 0.25 mM EDTA (pH 8), 1% Triton X-100, 0.2% sodium fluoride, and protease inhibitor mixture (Sigma)]. Equal amount of proteins was resolved on 7.5% SDS-polyacrylamide gel and blotted onto PVDF membrane. Western blotting was performed according to standard protocols. The p-JAK2 (cat. no. 3776), p-STAT3 (cat. no. 9145), p-STAT4 (cat. no. 5267), anti-rabbit IgG-HRP (cat. no. 7074), and p-CSFR (cat. no. 3155) were purchased from Cell Signaling Technology; p-STAT5 (cat. no. 3976), p-STAT5 (cat. no. 3512), anti-rabbit IgG-HRP (cat. no. 7074), p-CSFR (cat. no. 3512), anti-rabbit IgG-HRP (cat. no. 7076), and p-FLT3 (cat. no. 3461) Abs were from Cell Signaling Technology; p-STAT5 (cat. no. 611965) was from BD Biosciences (San Jose, CA); and actin Ab (cat. no. A2066) was from Sigma. The blots were developed using SuperSignal West Dura extended duration substrate (Thermo Scientific, Rockford, IL). The images were captured digitally using the LAS-3000 Life Science Imager, and densitometric analysis was performed using MultiGauge v3.1 software.
CD4+ T cells were activated with anti-CD2, anti-CD3, and anti-CD28 Ab-coated beads (bead-to-cell ratio 1:2; T cell activation/expansion kit; Miltenyi Biotec, Bergisch Gladbach, Germany) for 3 d in X-VIVO 20 media (Lonza, Basel, Switzerland) at a density of 0.5 × 10^6 cells/ml. After activation, the cells were differentiated for 4 d with IL-12 (20 ng/ml) and anti–IL-4 Ab (2.5 μg/ml) in titrating concentrations of SB1578. To detect intracellular cytokine expression, the cells were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma) for 6 h in the presence of Golgiplug. Intracellular cytokine staining with Alexa Fluor 488-labeled anti-collagen type II IgG in the serum were determined by ELISA using FlowJo software v7.6 (Tree Star, Ashland, OR).

Monocyte differentiation assay
Peripheral blood from healthy human donors was collected in Vacutainer CPT tubes (BD Biosciences), and mononuclear cells were isolated using density gradient centrifugation. Monocytes were isolated by negative selection using monocyte isolation kit II (Miltenyi Biotec). Purified monocytes were cultured for 7 d at a cell density of 3 × 10^5 cells/ml in RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM NEAA, 10 mM HEPES, and 50 ng/ml HGF (Cell Signaling Technology). Cells were harvested on day 7, washed with PBS containing 5% FBS, and stained with FITC-conjugated anti-CD14 Ab (BD Biosciences). Samples were acquired on a BD FACSCalibur, and the data were analyzed using FlowJo software v7.6.

Culture and differentiation of DCs
Total bone marrow cells were collected by flushing the tibiae and femora from the BALB/c mice. The lineage-negative (Lin−) cells were enriched by immunomagnetic depletion using mouse hematopoietic progenitor (stem) cell enrichment set (BD Biosciences). The Lin− cells were cultured at a density of 5 × 10^5 cells/ml in RPMI 1640 with 10% FBS, 2 mM l-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM NEAA, 1 mM sodium pyruvate, and 100 ng/ml L-glutamine (eBioscience, San Diego, CA). After 7 d, the cells were harvested and stained with PE-labeled CD11c Ab (BD Biosciences). The cells were collected on a FACSCalibur, and the data were analyzed using FlowJo software v7.6.

Pharmacokinetic analysis
Rat. Healthy male Lewis rats (200–250 g; Charles River Laboratories) were administered 2, 7, and 28 mg/kg SB1578 as a solution in PBS at a dose volume of 10 ml/kg twice daily i.p. for 4 d. Blood samples were collected from two rats per time point for each dose level on days 1 and 4 by tail vein bleeding at 0, 15, 30 min and 1, 2, 4, 8, and 24 h postdose. Plasma was obtained and analyzed using a validated bioanalytical LC/MS/MS method. Mice. BALB/c mice (18–20 g; Charles River Laboratories) were orally administered SB1578 at doses of 20, 70, and 210 mg/kg twice daily for 28 d at a dose volume of 10 ml/kg, and blood samples were collected on days 1 and 28. The samples were collected from cohorts of three animals at 0.5, 1, 2, 4, and 8 (day 1) and predose, 0.5, 1, 2, 4, 8, and 24 h (day 28) via cardiac puncture in anticoagulant. Plasma was obtained and analyzed using a validated bioanalytical LC/MS/MS method.

Measurement of cytokine levels
Blood samples were obtained from the mice by cardiac puncture at the termination of study, and serum was prepared. Levels of 32 cytokines were quantitated in the samples using MILLIPLEX MAP Mouse Cytokine Chemokine-Premixed 32x (MXPCYTOM32L1; Millipore). The 32 cytokines/chemokines analyzed are G-CSF, GM-CSF, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, KC, LIF, LIX, M-CSF, MCP-1, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, and VEGF. The levels of anti-collagen type II IgG in the serum were determined by ELISA using a kit from Chondrex (Redmond, WA) following the manufacturer's instructions. At study termination, knees from all the animals in the prophylactic protocol were flushed with 30 μl PBS, and the lavages were collected for cytokine analysis. Levels of IL-6 and IL-1β in the knee lavages were measured using cytokine ELISA kits (R&D Systems, Minneapolis, MN).

Macrophage staining
Macrophage infiltration in the hind paws of the mice was determined by immunostaining with F4/80 Ab. The sections were deparaffinized in xylene and rehydrated in a series of ethanol gradients. After Ag retrieval with proteinase K treatment, the endogenous peroxidase activity was blocked by incubation with 3% H2O2, and background signals were blocked by incubation with 10% goat serum. After an overnight incubation with an anti-F4/80 Ab (MCA497GA; AbD Serotec, Kidlington, U.K.) the sections were incubated with anti-rat IgG-HRP (Thermo Scientific). Incubation with 3,3’-diaminobenzidine (DAKO, Glostrup, Denmark) chromogen substrate resulted in brown staining of the F4/80+ cells. The nuclei were counterstained with Mayer’s hematoxylin before dehydration and mounting. Images of whole paws were captured on an automated imaging system at ×20 magnification, and the total number of F4/80+ cells in each paw was quantified using the analysis software from Ariol (Genetix USA, San Jose, CA).

Neutrophil staining
For neutrophils, formalin-fixed paraffin-embedded sections were stained with anti-neutrophil Ab (NIMP-R14; ab2557; Abcam, Cambridge, U.K.) followed by incubation with anti-rat IgG (B9468; DAKO) and subsequently anti-rabbit IgG-HRP (K4010; DAKO). 3,3’-Diaminobenzidine chromogen was used as a substrate for detection of positively stained cells, and sections were subsequently stained with hematoxylin. Neutrophil counts were done in sections by counting the number of neutrophils in a typical heavily infiltrated ×40 objective field and then estimating total number of fields present in the worst affected section of the paw.

Statistical analysis
Data were analyzed using Prism v5.0 software. Significance for all tests was set at p ≤ 0.05. The statistical test applied for each experiment is detailed in the respective figure legends.

Results
Kinase selectivity spectrum of SB1578
SB1578 originated from a series of novel pyrimidine-containing low m.w. macrocycles discovered in the S→BIO kinase inhibitor program (30, 31). As shown in Supplemental Table I, its potency against JAK2 (IC50 = 46 nM) was achieved with good selectivity against JAK1 and JAK3 (>60-fold) and 5-fold difference against TYK2 (IC50 = 230 nM). Upon further characterization of its broader kinase spectrum, SB1578 was found to be a potent inhibitor of FLT3 (IC50 = 62 nM) and c-Fms (IC50 = 69 nM). In addition, SB1578 was also tested against various kinases from a representative cross-section of the human kinome, and the various IC50 are shown in Supplemental Table I. SB1578 showed at least 5-fold selectivity between JAK2 and these other tested kinases.

SB1578 inhibits JAK2 signaling and prevents differentiation of Th1 cells
To determine if the observed kinase inhibitory activity of SB1578 translates into the modulation of JAK2-mediated signaling and function in the cells, we investigated the effect of SB1578 on IL-12–induced JAK2 phosphorylation in human CD4+ T cells. Binding of IL-12 to its receptor causes the autophosphorylation of JAK2 and activation of downstream STAT proteins, mainly STAT3 and STAT4 (32). Consistent with its biochemical kinase inhibition, SB1578 led to a concentration-dependent inhibition of JAK2 autophosphorylation (IC50 = 165 nM) (Fig. 1A). Consequently, the IL-12–stimulated phosphorylation of STAT3 and STAT4 was also inhibited with IC50 values of 124 and 139 nM, respectively (Fig. 1A). Additionally, treatment with SB1578 also decreased Tyk2 phosphorylation in the IL-12–stimulated T cells, consistent with the inhibition of purified Tyk2 kinase (Supplemental Fig. 1 and Supplemental Table I).
T cells play a key role in the initiation and progression of CIA by producing proinflammatory cytokines and chemokines, hence promoting infiltration and activation of macrophages and neutrophils (3). IL-12 is the key cytokine known to mediate Th1 cell differentiation through activation of the JAK–STAT signaling pathway, specifically via JAK2. As SB1578 prevents the IL-12–mediated phosphorylation of JAK2 and consequently STAT3 and STAT4, we analyzed its effect on differentiation of CD4+ T cells to Th1 subset. CD4+ T cells from three healthy donors were exposed to titrating concentrations of SB1578 during activation and differentiation with IL-12. Inhibition of JAK2 by SB1578 prevented the differentiation to Th1 cells, as measured by reduction in IFN-γ-producing cells after 4 d in culture (Fig. 1B). The effect of SB1578 on Th1 cell differentiation was dose dependent with an IC50 of 155 nM.

**SB1578 inhibits c-Fms signaling and prevents differentiation of macrophages**

In addition to JAK2 signaling, the effect of SB1578 on c-Fms signaling in a cellular context was also analyzed. THP-1 cells stimulated with M-CSF induced the autophosphorylation of c-Fms, which was inhibited on treatment with SB1578 (IC50 = 584 nM) (Fig. 1C). To determine whether c-Fms inhibition has an impact on the differentiation of macrophages, monocytes isolated from peripheral blood were differentiated with M-CSF in the presence or absence of SB1578. SB1578 suppressed the expression of...
differentiation-dependent Ag CD71 (33, 34) on the cells in a dose-responsive manner (IC50 = 277 nM, Fig. 1D).

**SB1578 inhibits FLT3 signaling and prevents DC differentiation**

As in vitro kinase profiling revealed SB1578 to be a potent FLT3 inhibitor, we studied its efficacy on signaling in the mouse bone marrow lineage-depleted cells (Lin-). Stimulation of these cells with FLT3L for 5 min induced the autophosphorylation of FLT3 (Fig. 1E), and this was inhibited by SB1578 (IC50 = 138 nM). Consequently, the FLT3L-induced phosphorylation of downstream mediators STAT3 and STAT5 was also inhibited with IC50 values of 211 and 52 nM, respectively. A large proportion of DCs are derived from the FLT3-expressing hematopoietic progenitors. To examine the effect of SB1578 on FLT3L-induced DC development, the Lin- cells were cultured with or without SB1578 for 7 d, and the percentage of DCs that develop was measured. We observed that SB1578 can inhibit the development of DCs (CD11c+ cells) in FLT3L-induced bone marrow cultures with an IC50 of 185 nM (Fig. 1F).

**SB1578 is efficacious in a rat model of AIA**

Rat adjuvant arthritis, an experimental model of polyarthritis, has been widely used for preclinical testing of numerous antiarthritic agents. To assess the potential of treatment with SB1578, male Lewis rats were administered twice daily i.p. SB1578 (7, 14, or 28 mg/kg) or 0.075 mg/kg methotrexate. The i.p. route was chosen because of the low oral bioavailability (6%) of SB1578 in rats. SB1578 showed rapid absorption, and mean steady-state peak concentrations exceeded the biochemical IC50 values at 7 and 28 mg/kg (Fig. 2A). At steady state, the concentrations were above IC50 values for all three kinase for at least 8 h at 7 mg/kg and at least 14 h at 28 mg/kg dose. Steady-state exposure was dose-proportional between 2 and 28 mg/kg.

The rats treated with SB1578 showed dose-dependent amelioration of clinical and histopathological symptoms associated with developing adjuvant arthritis compared with the vehicle controls, without a significant effect on the body weight (data not shown). Ankle diameter was reduced by 31% in rats treated with 28 mg/kg SB1578 compared with the vehicle controls (Fig. 2B), and AUC for the clinical paw scores was also decreased by 50% at this dose of SB1578 (data not shown). This reduction in ankle diameter was accompanied by the beneficial effects of SB1578 on the histopathological parameters of bone resorption and inflammation (Fig. 2C). Ankles from the vehicle control animal showed severe inflammation and synovitis with moderate bone resorption in distal tibial physis, whereas the ankles from rats treated with 28 mg/kg SB1578 had mild to moderate inflammation and synovitis with mild bone resorption in distal tibial physis. Thus, treatment with 28 mg/kg SB1578 led to a decrease in the inflammation (34%) and bone resorption (46%), contributing to a significant improvement of ankle histopathology (Fig. 2D). However, the rats treated with methotrexate (MTX) had significant improvement in ankle histopathology with minimal inflammation and synovitis.

The use of an adjuvant model also offers an opportunity to study pathological changes in spleen and liver. Splenomegaly occurs as a result of increased extramedullary hematopoiesis (EMH) in the red pulp in conjunction with polygranulomatous inflammation in the red pulp and capsule (35). A significant effect on splenomegaly was observed in the rats of the 28 mg/kg treated group with a reduction in the inflammation (79%), EMH scores (68%), and lymphoid atrophy (30%) compared with the vehicle group, which showed marked inflammation, EMH, and moderate lymphoid atrophy (Fig. 2E, 2F). The effect of SB1578 was superior to MTX in terms of the resolution of splenomegaly where the excess spleen weights were reduced by 82% with 28 mg/kg SB1578 compared with 52% with MTX (Fig. 2G). Hepatomegaly was also resolved as the liver weight was normalized by more than 50% on treatment with 28 mg/kg SB1578 compared with the vehicle treated rats (Fig. 2H).

**Amelioration of CIA in mice with oral administration of SB1578**

In addition to the AIA model, the potential of treatment with SB1578 in RA was explored using the CIA model in mice. Clinical signs and histological developments in the murine CIA model resemble those of human RA and involve both cellular and humoral immune responses during disease progression. Not only does the model recapitulate hallmark symptoms of human RA such as joint inflammation, cartilage destruction, and bone resorption, but the study animals also frequently present with systemic inflammatory symptoms such as spleen enlargement and elevated cytokine levels.

Oral pharmacokinetics of SB1578 in mice was characterized by dose-proportional increase in exposures between 20 and 210 mg/kg, and the steady-state concentrations were above all three kinase IC50 values at 20, 70, and 210 mg/kg on day 1 and day 28 (Supplemental Fig. 2). On the basis of the pharmacokinetic profiling in mice (Supplemental Fig. 2), CIA mice were administered 70, 140, and 210 mg/kg SB1578 twice daily. After immunization with collagen, the first signs of the disease were apparent on day 18 (Fig. 3A). All vehicle control mice had the disease by day 22 and reached a clinical score of 1.45 ± 0.3, which gradually increased to a maximum of 2.75 ± 0.4 on study termination at day 28. Prophylactic treatment at all doses of SB1578 suppressed arthritis development. Oral administration at 70 mg/kg reduced the disease incidence by 70%, and the clinical scores were reduced to 0.63 ± 0.37 on day 28. Treatment at a higher dose of 140 mg/kg decreased disease incidence by 80% and was accompanied by a further reduction of clinical score to 0.13 ± 0.1 at day 28. The highest dose of SB1578 (210 mg/kg) completely abrogated disease development in all the mice, with no disease incidence at study termination. Daily clinical arthritis scores expressed as AUC were significantly decreased in a dose-responsive manner with a 79% reduction observed even at the lowest dose of 70 mg/kg and 100% reduction in the group treated with 210 mg/kg SB1578 (data not shown). Body weight was not significantly affected for mice in any treatment group compared with the vehicle controls (data not shown).

Histopathological examination of the arthritic joints (ankles, knees, and digits) of the untreated mice revealed several changes that are consistent with those seen in type II CIA. Microscopic alteration included massive infiltration of the synovium and periarticular tissue with inflammatory cells (inflammation), extensive cartilage damage (chondrocyte loss and collagen matrix destruction), mild to moderate marginal zone pannus, presence of osteoclasts, and bone resorption (Fig. 3B). Mice treated with SB1578 showed significant dose-dependent reduction in the six-joint histopathology score for all the above parameters (Fig. 3C). Even the lowest dose of 70 mg/kg SB1578 resulted in a 72% reduction of the composite score that includes inflammation, cartilage damage, bone resorption, and pannus formation. A reduction of 89% was observed with 140 mg/kg and a complete restoration to normal with a dose of 210 mg/kg SB1578, which was comparable to dexamethasone treatment.

The beneficial effect of SB1578 treatment was also extended to resolution of splenomegaly in the murine CIA model, in line with the observation in the rat AIA model. Compared with the vehicle group, the spleen weights were restored to normal in the mice treated with SB1578 (Fig. 3D).
FIGURE 2. SB1578 prevents adjuvant-induced arthritis in rats. (A) Mean plasma concentration versus time profiles of SB1578 in healthy male Lewis rats after i.p. administration at 2, 7, and 28 mg/kg twice daily for 4 d. (B) Mean ankle diameter of rats. Arthritis was induced in rats by injection of lipoidal amine, and twice-daily dosing with SB1578 (7, 14, 28 mg/kg or 0.075 mg/kg MTX) was initiated on day 0; \( n = 8 \) for all groups except naive \( (n = 4) \). Values shown are mean ± SEM. (C) Histopathological evaluation of the arthritis-induced damage to the ankles of rats: representative images of H&E-stained sections from the ankles of rats visualized at \( \times 16 \) magnification. Synovium is indicated by “S”; arrows indicate bone resorption in the distal tibial physis. (D) Bars represent sum of histopathology scores including inflammation and bone resorption of the ankles from animals of different groups. *\( p \leq 0.05 \) (t test to vehicle). (E) Representative images of H&E-stained sections from the spleens of the rats visualized at \( \times 50 \) magnification. EMH in the red pulp is shown by the arrow. R, Red pulp; W, white pulp; I, inflammation. (F) Histopathological evaluation of spleen. The bars represent scores for inflammation, lymphoid atrophy, and EMH in the spleens of the mice from all six groups. Data are represented as mean ± SEM. (G) Spleen weights relative to their body weight; data are represented as mean ± SEM. (H) Mean liver weights relative to their body weight; data are represented as mean ± SEM. *\( p \leq 0.05 \) (t test to vehicle).
We extended our study to assess the efficacy of SB1578 using a therapeutic protocol in the CIA model. SB1578 was administered orally twice daily to the mice only after the onset of disease (starting days 18–22), which was evident by swelling in at least one paw, and the treatment was continued for 10 d postenrollment. In the vehicle group, the clinical arthritic score rapidly increased from 0.52 ± 0.2 on disease day 1 to 2.8 ± 1.2 on disease day 11 (Fig. 4A). SB1578 treatment led to a dose-dependent inhibition of the clinical arthritic scores. The scores were only 2.15 ± 0.8 on day 11 in the mice administered 105 mg/kg SB1578. Notably, in the mice treated at 210 mg/kg, the effect was comparable to that of the potent corticosteroid dexamethasone, with the clinical arthritic scores significantly reduced to 1.05 ± 0.5.

Histopathological analysis of the sections from the mice of the vehicle group shows marked inflammation, moderate cartilage damage, pannus formation, and bone damage in the ankle and most digit joints (Fig. 4B). Additionally, surfaces with total cartilage loss in the knees were also observed. Similar to the findings in the prophylactic model, even when the treatment was initiated after the onset of disease, SB1578 (210 mg/kg) had beneficial effect and significantly improved the histopathology of the paws, knees, and ankles. There was a reduction in the number of affected joints as well as the extent of inflammation and cartilage or bone damage in the affected joints. We observed a decrease in inflammation score by 42% in the joints of the 210 mg/kg group. In addition, a 48% reduction in the surfaces with complete cartilage loss as well as diminished intensity of cartilage damage was detected. Pannus formation and damage to the bones was also reduced by 57 and 59%, respectively (Fig. 4C). Consequently, treatment with SB1578 significantly reduced the composite score for all joint histopathology parameters to 50%, accompanied by substantial abrogation of the characteristic CIA in SB1578-treated mice.

SB1578 treatment prevents macrophage and neutrophil infiltration

Macrophages are one of the major sources of inflammatory mediators in arthritis, and a decrease in their numbers in the joints correlate with clinical improvement of the disease (36). As treatment with SB1578 significantly decreased inflammation of the joints, we examined the effect of SB1578 on macrophage infiltration. For this, the paw sections of mice were stained for F4/80, a macrophage marker. As shown in Fig. 5A and 5B, pronounced infiltration of the macrophages in the synovial lining of the joints in the untreated CIA mice was evident upon enumeration of the total number of F4/80+ cells (11.8 × 10^6 cells) in both the hind paws compared with the naive mice (0.5 × 10^6 cells).
Notably, in the paws of mice treated with 210 mg/kg SB1578, there was a dramatic reduction in the number of infiltrating macrophages (0.3 ± 10⁴ cells), which constitutes the baseline in the naive mice.

In addition to macrophages, neutrophils are implicated in both the onset and progression of arthritic disease, particularly in the process of joint degradation (37, 38). To analyze the infiltration of neutrophils, the joints of the CIA mice were stained with anti-neutrophil Ab. Whereas there were negligible numbers of neutrophils in the paws and knees of the naive mice, the joints of the CIA mice showed massive influx of neutrophils (Fig. 5C). Treatment with 210 mg/kg SB1578 led to a significant decrease in the numbers of infiltrating neutrophils. Thus, SB1578 treatment not only prevents the infiltration of macrophages but also affects additional cell populations like neutrophils, enhancing its effects on the histological improvement of the joints.

SB1578 treatment does not inhibit collagen-specific humoral immunity

High levels of circulating Abs against the collagen-rich joints accompany the development of RA and CIA. Abs to type II collagen were analyzed in the sera obtained from the CIA mice at the end of the study. There was no difference in the serum levels of type II collagen IgG between the 210 mg/kg SB1578-treated group (9.49 mg/ml) and the vehicle group (9.54 mg/ml) (Fig. 5D). However, the dexamethasone-treated mice showed reduced levels (4.9 mg/ml) of type II collagen IgG.

SB1578 treatment reduces the levels of proinflammatory cytokines

To investigate how proinflammatory cytokines are affected by SB1578 treatment in the CIA model, we analyzed the levels of the cytokines linked to disease severity in RA. In concordance with the observed inflammation, the levels of proinflammatory cytokines IL-1β and IL-6 were found to be significantly elevated in the affected knee joints of the diseased mice relative to the naive mice. The mean levels of IL-1β and IL-6 were increased to 700 pg/ml and 1300 pg/ml, respectively, compared with undetectable levels in the naive mice. Treatment with 210 mg/kg SB1578 completely abrogated this increase (Fig. 6A, 6B) with the levels of both these cytokines restored to levels indistinguishable from the naive animals.

To assess the changes in other cytokine levels, we measured 32 different cytokines and chemokines in the serum of the mice using a multiplex assay. The analysis revealed that similar to the knee lavages, levels of IL-6 were significantly elevated in the serum of mice from the vehicle group (>100-fold) (Fig. 6C). Additionally, KC, a neutrophil chemoattractant, was also elevated by 300-fold in the diseased mice (Fig. 6D). Treatment with 210 mg/kg SB1578 restored the levels of both these cytokines to normal. The levels of monokine induced by IFN-γ (MIG) and IL-9 were also altered in the diseased mice, and treatment with SB1578 normalized their levels (Supplemental Fig. 3); however, this effect was not statistically significant. The levels of IL-1α, IL-13, Eotaxis, IP-10, LIX, RANTES, MCP-1, MIP-1β, MIP-1α, and G-CSF were un-
altered among the three groups. Additionally, the cytokines GM-CSF, IFN-γ, M-CSF, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-17, IL-10, IL-12(p70), IL-15, IL-17, MIP-2, LIF, TNF-α, IL-12(p40), VEGF, and IL-7 were below the detection limit.

Discussion

In this study, we show that SB1578 is a novel, orally available kinase inhibitor that shows remarkable potency not only in preventing the development of experimental arthritis but also in its treatment after disease onset. It has a unique kinase spectrum and selectively inhibits JAK2, c-Fms, and FLT3, three kinases that are critical to the pathogenesis of arthritis, which is unprecedented in the current stable of kinase inhibitors (39). It is effective in suppressing many of the manifestations of the disease including joint swelling, cytokine secretion, and inflammatory cell infiltration in the synovial joints and most importantly the destruction of bones.

**FIGURE 5.** SB1578 treatment reduces the infiltration of macrophages and neutrophils in the murine CIA model. (A) Representative images of the section from hind paws of mice from naive, vehicle, and SB1578 (210 mg/kg twice daily) treated groups stained with F4/80 Ab. The upper panels show an overview (scale bars, 2.7 mm), and a detailed view is shown in the lower panels (scale bars, 200 μM). (B) Total number of F4/80⁺ cells were counted in the paw sections using Ariol imaging and analysis software. (C) Neutrophil infiltration in the joints as determined by enumerating the cells positively stained with anti-neutrophil Ab. (D) The levels of Abs against type II collagen were determined in the serum from the mice of the developing disease model by ELISA. Data were analyzed using Kruskal–Wallis test followed by Dunn’s multiple comparison test and are presented as mean ± SEM. *p < 0.05.

**FIGURE 6.** Effect of SB1578 treatment on cytokine levels in the serum and knee lavages of CIA mice. Cytokine levels in the knee lavages [(A) and (B)] or sera [(C) and (D)] of mice from the naive, vehicle, and SB1578 (210 mg/kg twice daily) treated groups of the developing disease model were measured as described in Materials and Methods. Data were analyzed using Kruskal–Wallis test followed by Dunn’s multiple comparison test. *p < 0.05.
and cartilage. Our data indicate that SB1578 potently inhibits diverse cellular responses that are primary drivers of RA pathogenesis. Our results from the rat AIA and murine CIA models suggest that with the kinase profile of SB1578, inhibition of JAK1 and JAK3 is not mandatory for therapeutic benefit in RA.

JAK2 is a key component in the signaling pathways elicited by proinflammatory cytokines such as IFN-γ, IL-6, IL-12, and IL-23 that play a fundamental role in the inflammatory process and articular destruction in arthritis (5, 40). These cytokines in combination with other factors initiate and sustain the T cell and B cell activation leading to production of proteases and other mediators that specifically regulate the inflammatory response, tissue damage, and remodeling processes.

The levels of proinflammatory cytokines IL-1β and IL-6 were highly elevated in synovial fluid and/or serum from the CIA mice. Both cytokines are of pivotal importance in RA as their levels in serum and synovial fluid correlate with disease progression and joint destruction in RA patients (7, 41). Improvements in clinical symptoms, including disease development and joint damage, obtained with the Ab against IL-6 receptor, tocilizumab, and human IL-1 receptor antagonist, anakinra, validate the concept for targeting these cytokines in antiarthritic therapy (8, 42). Notably, inhibition of JAK–STAT pathway by SB1578 strongly reduced the inflammatory response by reducing the levels of both these clinically validated therapeutic targets in the synovial joints and/or serum of the SB1578-treated arthritic mice. The levels of IL-6 and IL-1β were restored to the levels observed in naive mice, and this was accompanied by a decrease in disease severity including reduction in joint inflammation and concomitant histological improvement.

In addition to the proinflammatory cytokines, serum levels of the chemokine KC, a neutrophil chemoattractant, were significantly reduced upon treatment with SB1578. Elevated systemic levels of KC precede the onset of the disease in rats, and intra-articular increase in its level is associated with the progression of joint erosions during CIA (6). Neutrophils constitute a predominant leukocyte population in the synovial fluid of patients with inflammatory arthritis such as RA. They play an essential role in initiation of joint-specific inflammation and contribute to the bone and cartilage damage by releasing granules containing proteolytic enzymes and proinflammatory cytokines (38, 43). Neutrophil reduction is protective in rat AIA and murine CIA models. As shown in our study, the infiltration of neutrophils was elevated in arthritic joints of the CIA mice and was significantly reversed in the SB1578-treated mice, hence mitigating damage to the joints. The putative mechanism for the decrease in neutrophil infiltration could be the attenuated levels of KC. Administration of Abs to CXCR2, the receptor for KC, completely inhibits arthritis development in IL-1ra(−/−) mice by preventing the sustained infiltration of CXCR2-expressing neutrophils into the joints (44).

Signaling of some cytokines involved in the activation of B cells is also mediated via the JAK–STAT pathway. However, the formation of anti-collagen IgG was not affected in the sera of SB1578-treated mice. Thus, modulation of humoral immunity may not contribute to the observed therapeutic effects of SB1578 in the CIA model, consistent with two other reported JAK2 inhibitors (45, 46).

Complementing the anti-inflammatory property of SB1578 is its ability to modulate the autoimmune component of the disease. The earlier events in the development of RA are initiation and establishment of autoimmunity to collagen-rich joint components followed by destructive inflammatory responses. The induction of the autoimmune response in CIA involves the development of autoreactive Th1 cells, their migration into the joint tissues, and subsequent recruitment of inflammatory cells through multiple mediators (47). In addition to reducing the proinflammatory cytokines, SB1578 prevents the IL-12–mediated signaling as demonstrated by decreased phosphorylation of STAT3 and STAT4 in CD4+ T cells. This prevents the differentiation of naive Th cells into Th1 subtype and provides a highly effective therapy for CIA by reducing the levels of IFN-γ, a potent inducer of inflammatory response.

Targeted inhibition of APCs is an alternative to direct inhibition of T cell responses for the treatment of autoimmun diseases (48). APCs, such as macrophages and DCs, are abundant in the rheumatoid synovial tissues and by presenting autoantigens locally, they lead to amplification of systemic arthritogenic immune responses (49). Signaling via FLT3, expressed on DC progenitors and mature DCs, results in increased numbers of functional DCs and is required for their survival and activation (27). Inhibition of FLT3 signaling and DC differentiation by SB1578 potentiates its antiarthritic activity by suppressing the presentation of autoantigens. We observed that DC populations are reduced in the spleens of SB1578-treated CIA mice (data not shown). Sunitinib, a potent FLT3 inhibitor, has been demonstrated to have potential for the treatment of arthritis by decreasing DC numbers and function (48, 50).

Notably, the capacity of SB1578 to inhibit c-Fms adds a distinctive dimension to its profile. Signaling via c-Fms mediates the differentiation of monocytes to macrophages and osteoclasts (24). Macrophages are aberrantly activated in RA and play a vital role in chronic synovial inflammation. Upon activation, they synthesize mediators such as the eicosanoids, PGE2, and the cytokines TNF-α and IL-1β that induce the production of a variety of enzymes involved in cartilage and bone destruction (51). By virtue of its ability to prevent c-Fms signaling, it is conceivable that SB1578 prevents the differentiation of monocytes into macrophages, resulting in a decrease in absolute macrophage numbers that infiltrate into the paws of the CIA mice. The c-Fms–specific inhibitor GW2580 has been shown to exert therapeutic effects in murine CIA models by preventing macrophage and osteoclast differentiation (24). Synovial tissue macrophages are a sensitive biomarker in patients with RA, and reduction in their numbers correlates with clinical improvement in RA (36). This was also observed on treatment with SB1578, which decreases macrophage infiltration and alleviates cartilage destruction, leading to clinical improvement and improvement in pathology in CIA. The beneficial effect of SB1578 treatment on bone erosion was less profound compared with MTX. This could likely be due to the fact that inflammation, bone erosion, and cartilage erosion are separate processes regulated by independent mechanisms, potentially needing selective therapy.

We postulate that the high efficacy of SB1578 observed in the mouse model of RA can be attributed to its inhibition of signaling via JAK2, c-Fms, and FLT3 that together drive pathological processes in RA. SB1578 not only downregulates the production of inflammatory mediators and inhibits infiltration of pathological cell populations such as neutrophils but also suppresses the autoimmune response by preventing the differentiation of Ag-presenting DCs and generation of autoreactive T cells. It also inhibits monocyte differentiation, leading to reduced macrophage numbers in the joints. This combined effect of SB1578 on three different signaling cascades is the probable mechanism for the prevention of synovial hyperplasia and bone destruction, a prominent feature of RA. While most antiarthritis drugs such as MTX, corticosteroids, and nonsteroidal anti-inflammatory drugs can suppress inflammation and pain, only a few agents are capable of slowing bone resorption and cartilage damage. Although biologics like TNF-α, IL-1β, and IL-6 blockers have revolutionized the treatment of RA, a substantial
number of patients fail to respond adequately or suffer from serious side effects (52). As cytokines exert a synergistic effect in RA, the use of kinase inhibitors that demonstrate wide-ranging effects on inflammatory mediators may give a broader patient response than biologic agents that work through single targets. Small-molecule inhibitors of JAKs are emerging as promising therapies for RA. Two compounds, tofacitinib (JAK1/2/3 inhibitor) and LY309014 (JAK1/2 inhibitor), are undergoing late-stage clinical trials or nearing U.S. Food and Drug Administration approval for marketing (45, 53, 54). The combination of activities of SB1578 against JAK2, c-Fms, and FLT3, with selectivity for JAK2 within the JAK family, is unique. The lack of JAK1 and JAK3 inhibition may confer a slightly different immunomodulation profile for SB1578. The activities against these three kinases, which play crucial roles in the pathogenesis of arthritis, differentiates SB1578 from other JAK family kinase inhibitors that are in clinical trials for RA and provides a novel and attractive therapeutic alternative.

To conclude, SB1578 belongs to a new class of orally bioavailable small-molecule drugs that is efficacious and well tolerated in preclinical models of arthritis. Its favorable pharmacokinetic properties and safety profile provide a rationale for clinical development in a wide range of inflammatory autoimmune diseases, including RA, inflammatory bowel disease, and psoriasis. In the phase I clinical trials in healthy volunteers (NCT01235871; www.clinicaltrials.gov), the safety and pharmacokinetics of SB1578 have been evaluated, and it is well tolerated (no significant hematologic toxicity) in the dose range 10–50 mg/kg. The next pertinent course of action is to determine the pharmacodynamic responses in this dose range.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


Figure S1: SB1578 blocks the TYK2 phosphorylation: CD4\(^+\) T cells were incubated with various concentrations of SB1578 for 3 h followed by stimulation with IL-12 (100 ng/mL) for 10 min. After lysis, pTYK2 (Y1054/1055) was detected by immunoblotting, with actin as a loading control.
Figure S2: Pharmacokinetic profiling of SB1578 in mice: Plasma concentration v/s time profiles of SB1578 in healthy male Balb/c mice following oral doses of 20, 70 and 210 mg/kg on day 1 (a) and day 28 (b). Data are represented as mean ± SEM.
Figure S3: Effect of SB1578 treatment on MIG and IL-9 levels in the serum of CIA mice:

Cytokine levels in the sera of naïve, vehicle and SB1578 (210 mg/kg b.i.d) treated mice were measured as described in the ‘Material and Methods’. Data were analyzed using Kruskal-Wallis test followed by Dunn’s multiple comparison test.
Table S1. Structure and in vitro Kinase Spectrum of SB1578.

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<td>(9E)-15-(2-Pyrrolidin-1-yl-ethoxy)-7,12,25-trioxa19,21,24triazatetracyclo[18.3.1.1(2,5).1(14,18)]hexacosa-1(24),2,4,9,14,16,18(26),20,22 nonaene</td>
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