R406, an Orally Available Spleen Tyrosine Kinase Inhibitor Blocks Fc Receptor Signaling and Reduces Immune Complex-Mediated Inflammation

Sylvia Braselmann, Vanessa Taylor, Haoran Zhao, Su Wang, Catherine Sylvain, Muhammad Baluom, Kunbin Qu, Ellen Herlaar, Angela Lau, Chi Young, Brian R. Wong, Scott Lovell, Thomas Sun, Gary Park, Ankush Argade, Stipo Jurcevic, Polly Pine, Rajinder Singh, Elliott B. Grossbard, Donald G. Payan, and Esteban S. Masuda


Received June 5, 2006; accepted August 30, 2006

ABSTRACT

Recent compelling evidence has lead to renewed interest in the role of antibodies and immune complexes in the pathogenesis of several autoimmune disorders, such as rheumatoid arthritis. These immune complexes, consisting of autoantibodies to self-antigens, can mediate inflammatory responses largely through binding and activating the immunoglobulin Fc receptors (FcRs). Using cell-based structure activity relationships with cultured human mast cells, we have identified the small molecule R406 as a potent inhibitor of Fc receptor signaling (EC50 for degranulation = 56–64 nM). Here we show that the primary target for R406 is the spleen tyrosine kinase (Syk), which plays a key role in the signaling of activating Fc receptors and the B-cell receptor (BCR). R406 inhibited phosphorylation of Syk in human disease.

Rheumatoid arthritis (RA) is a chronic inflammatory disease of peripheral joints characterized by autoantibody expression, synovial inflammation, pannus formation, and erosion of cartilage and bone (Firestein, 2003). Despite significant efforts and advances, the etiology of RA and its pathogenesis remain incompletely understood, and the available medical treatments have limited efficacy. Thus, additional therapies with tolerable side effects are desired (Firestein, 2003; Smolen and Steiner, 2003).

Current opinion purports that in genetically susceptible patients, joint inflammation in RA is triggered by the activation of innate immunity with infectious or environmental

sions of cartilage and bone (Firestein, 2003). Despite significant efforts and advances, the etiology of RA and its pathogenesis remain incompletely understood, and the available medical treatments have limited efficacy. Thus, additional therapies with tolerable side effects are desired (Firestein, 2003; Smolen and Steiner, 2003).

Current opinion purports that in genetically susceptible patients, joint inflammation in RA is triggered by the activation of innate immunity with infectious or environmental

ABBREVIATIONS: RA, rheumatoid arthritis; FITC, fluorescein isothiocyanate; ITAM, immunoreceptor tyrosine-based activation motifs; Syk, spleen tyrosine kinase; CHMC, cultured human mast cells; R406, N4-(2,2-dimethyl-3-oxo-4H-pyrid[1,4]oxazin-6-yl)-5-fluoro-N2-(3,4,5-trimethoxyphenyl)-2,4-pyrimidinediamine; DMSO, dimethyl sulfoxide; LPS, lipopolysaccharide; Ig, immunoglobulin; IC, immune complex; CAβA, collagen antibody-induced arthritis; FcRI, receptor for Fc portion of immunoglobulins; IFN-γ, interferon-γ; TNFα, TNF, tumor necrosis factor α; PMA, phorbol 12-myristate 13-acetate; TLR, Toll-like receptor; LTC4, leukotriene C4; GM-CSF, granulocyte-macrophage colony-stimulating factor; BCR, B-cell receptor; OVA, ovalbumin; PEG, polyethylene glycol; LAT, linker for activation of T cells; IL, interleukin; FcεRI, high-affinity type-I receptor for IgE; Fli3, Fms-like tyrosine kinase 3; TPGS, δ-α-tocopheryl-polyethylene glycol-1000 succinate.
products and perpetuated with self-sustaining autoimmune responses. RA has been associated with several types of autoantibodies directed against antigens, such as type II collagen, heat-shock proteins, BiP, hnRNp-33, and citrullinated proteins, as well as with rheumatoid factors that recognize the Fc fragment of immunoglobulin (Ig) G (Steiner and Smolen, 2002). Thus, in this context, locally produced specific autoantibodies and immune complexes (ICs) can activate sentinel and effector cells, leading to inflammatory mediator release, and cytokine production, promoting further deleterious responses resulting in synovitis and joint structural damage.

The role for ICs in RA has been bolstered by the fact that, in mice, joint arthritic pathology can be elicited with the passive transfer of antibodies directed against glucose 6-phosphate isomerase in the K/BxN model or against collagen type II in the collagen antibody-induced arthritis (CAIA) model (Terato et al., 1992; Ji et al., 2002; Firestein and Corr, 2005). These antibodies and their ICs engage and activate Fc receptor (FcR) signaling in macrophages, neutrophils, and dendritic and mast cells, resulting in degranulation and cytokine gene transcription, which is critical for disease initiation and propagation. Importantly, synovial and articular inflammation induced by these antibodies was markedly suppressed in mice deficient in the FcγR signaling, the signaling component of activating Fcγ receptor complexes (Ji et al., 2002; Kagari et al., 2003). Thus, blocking this FcR signaling represents an attractive strategy for therapeutic intervention of IC-mediated inflammation.

FcγR signal transduction is critically dependent on immunoreceptor tyrosine-based activation motifs (ITAMs) located in its cytoplasmic tail (Berton et al., 2005; Nimmerjahn and Ravetch, 2006). Spleen tyrosine kinase (Syk), expressed predominantly in hematopoietic cells, binds these phosphorylated ITAM motifs and activates the events needed for downstream signaling. Syk is also essential for signal transduction of ITAMs, which are present among others in the B-cell receptor, the natural killer cell DAP12 receptor, and the platelet glycoprotein VI receptor (Turner et al., 2000; Wong et al., 2004; Berton et al., 2005). Thus, Syk is a central player in the activation of immune cells probably playing critical roles in the pathogenesis in RA.

Here we describe the identification and biological characterization of R406 as a small molecule inhibitor that primarily targets Syk. We show that R406 is orally available and potently inhibits IgE- and IgG-mediated activation of Fc receptor signaling. R406 inhibits the ITAM-dependent signaling by the Fc receptor complexes in macrophages, neutrophils and mast cells, and the ITAM-dependent signaling from the B-cell receptor. In mice, administration of R406 reduced vascular leakage and edema in the Arthus reaction elicited by immune complexes. R406 also showed efficacy in inhibiting paw inflammation in two antibody-induced arthritis mouse models. Finally, we report a first-in-human study with R406; in addition to preliminary observations on safety and pharmacokinetics, we assessed the effects of R406 on basophil activation and platelet aggregation.

**Materials and Methods**

**R406.** The small molecule N4-(2,2-dimethyl-3-oxo-4H-pyridin-1(4H)-oxazin-6-yl)-5-fluoro-N2-(3,4,5-trimethoxyphenyl)-2,4-pyrimidine-diamine (R406) was synthesized by the Department of Chemistry of Rigel Pharmaceuticals, Inc. The structure of R406 is shown in Fig. 1e. The discovery and structure-activity relationship leading to R406 will be presented elsewhere (R. Singh, manuscript in preparation).

**Human Mast Cell Culture and Stimulation.** Cultured human mast cells (CHMC) were derived from cord blood CD34+ progenitor cells and grown, primed, and stimulated as described previously (Rossi et al., 2006) and shown in supplemental data. Before stimulation, cells were incubated with R406 or DMSO for 30 min. Cells were then stimulated with either 0.25 to 2 mg/ml anti-IgE or anti-IgG (Bethyl Laboratories, Montgomery, TX) or 2 μM ionomycin (VWR, West Chester, PA). For tryptase measurement, ~1500 cells per well were stimulated for 30 min in modified Tyrode's buffer. For LTC4 and cytokine production, 100,000 cells per well were stimulated for 1 or 7 h, respectively. Tryptase activity was measured by luminescence readout of a peptide substrate, and LTC4 and cytokines were measured using LumineX multiplex technology.

**Western Blot.** Cells were preincubated at 1 x 10^6 cells/ml with DMSO vehicle or R406 at different concentrations for 40 min and stimulated with 2 μg/ml anti-human IgE or 5 min, or as indicated in the figure legend. The cells were spun down, washed in phosphate-buffered saline, and resuspended in Tris-glycine SDS sample buffer. For immunoprecipitations, 10 x 10^6 cells/ml were stimulated as described above and precipitated according to standard protocol. Western blots were performed according to standard protocol using 8% Tris-glycine gels, Immobilon P membrane, and ECL Western blot detection reagent. Primary antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA). Membranes were reprobed with antibodies recognizing various other proteins to verify equal amounts of protein in each lane.

**In Vitro Fluorescence Polarization Kinase Assay and Ki Determination.** The fluorescence polarization reactions were performed as described elsewhere (see supplemental data). For Ki determination, duplicate 200-μl reactions were set up at eight different ATP concentrations from 200 μM (2-fold serial dilutions) in the presence of either DMSO or R406 at 125, 62.5, 31.25, 15.5, or 7.8 nM. At different time points, 20 μl of each reaction was removed and quenched to stop the reaction. For each concentration of R406, the rate of reaction at each concentration of ATP was determined and plotted against the ATP concentration to determine the apparent Ki and Vmax (maximal rate). Finally the apparent Km (or apparent Kmax/Vmax) was plotted against the inhibitor concentration to determine the Ki. All data analysis was performed using Prism and Prism enzyme kinetics programs (GraphPad Software, Inc., San Diego, CA).

**Crystallography.** The kinase domain of Syk encompassing amino acid residues Ile358 to Asn635 with the single mutation E440Q as the search model. The kinase domain was built from the Advanced Photon Source Structural Biology Center Beamlne 19-BM at Argonne National Laboratory (Argonne, IL) using a custom built CCD detector and 1 degree omega-scans at a wavelength of 0.97934 Å. Indexing indicated a primitive orthorhombic lattice with a = 39.94 Å, b = 85.24 Å, and c = 91.02 Å, and the space group was determined to be P212121. The structure was solved by molecular replacement using a previously determined crystal structure of Syk kinase E440Q as the search model.

**Stimulation of Macrophages Derived from Primary Monocytes with IgG or LPS.** Human primary macrophages were derived from CD14- peripheral blood mononuclear cell (PBMCs; LLC, Emeryville, CA) according to the protocol specified in the monocyte isolation kit (Milteny Biotec, Auburn, CA) and by subsequently expanding the monocytes in 100 ng/ml human GM-CSF for 5 days to drive differentiation to macrophages (Munn and Cheung, 1989). THP-1 cells (ATCC, Manassas, VA) cells were primed with 10 ng/ml IFN-γ for 6 days before stimulation. Monocyte-derived macrophages were stimulated by immobilized (plate-bound) human IgG (Marsh et al., 1997). R406 and 15,000 cells were added to the IgG-coated wells.
and incubated for 16 to 20 h at 37°C. LPS was used at a final concentration of 10 ng/ml in uncoated wells. TNF concentration in the supernatants was measured by Luminex assay (Luminex Corp., Austin, TX).

**Stimulation of Primary Neutrophils with IgG or Phorbol 12-Myristate 13-Acetate.** Primary human neutrophils were isolated from heparinized human peripheral blood following Ficoll-Hypaque gradient separation and red blood cell lysis. Neutrophils were preincubated with R406 or DMSO in phosphate-buffered saline (PBS) for 20 min at room temperature. The cells were primed with TNFα (20 ng/ml) and stimulated for 15 min at 37°C with rabbit anti-human IgG (0.4 μg/ml), or phorbol 12-myristate 13-acetate (PMA, 0.1 μM). Respiratory burst activity was measured by adding dihydrorhodamine 123 to a final concentration of 0.5 μM and incubated for 10 min at 37°C. The cells were fixed, and intracellular fluorescence was analyzed by flow cytometry.

**Stimulation of Primary B Cells with Anti-IgM.** Primary human B cells were isolated from peripheral blood by Ficoll-Hypaque gradient separation followed by positive selection using CD19-coated magnetic beads according to manufacturer’s instruction (Dynal BioTech, Lake Success, NY). Alternatively, purified primary B cells were obtained from AllCells, LLC. Isolated B cells (65,000/well) were preincubated for 60 min with R406 in RPMI 1640 medium + 10% fetal bovine serum. Cells were stimulated with anti-IgM (5 μg/ml) for 6 h, stained with anti-CD69APC, and analyzed by flow cytometry.

**Microbicidal Activity of Neutrophils.** Neutrophils purified from human peripheral blood were pretreated with R406 or DMSO for 1 h at 4°C and then mixed with opsonized *Staphylococcus aureus* bacteria in a 1:1 ratio. The samples were rotated at 37°C, and at the indicated times, aliquots were plated onto blood agar plates and cultured overnight. Colony counts relative to the 0-min time point were used to determine the percentage of bacterial growth. The positive control, cytochalasin D, inhibits neutrophil microbicidal activity as shown by robust bacterial growth.

**Animals.** Female Balb/c mice (Hilltop Laboratory Animals, Inc., Scottsdale, PA) age 6 to 8 weeks were used in the CAIA studies. Female C57BL/6 mice were used in the Arthus studies and in the
K/BxN model performed by Dr. Diane Mathis at the Joslin Diabetes Center (Harvard Medical School, Boston, MA). All animals used in these studies were under the protocols approved for each model by each Institutional Animal Care and Use Committee.

**Arthus Reaction.** Reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Mice were challenged intravenously with 1% ovalbumin (OVA) in saline (10 mg/kg) containing 1% Evans blue dye. Ten minutes later, mice were anesthetized with isoﬂuorane and shaved dorsolaterally. The rabbit anti-OVA IgG (50 μg/25 μl) was injected intradermally on the left side of the back at three adjacent locations. Three injections of rabbit polyclonal IgG (50 μg/25 μl) on the opposite side of the same animal served as controls. R406 or vehicle (67% PEG 400) was administered to animals 60 min before antibody/antigen challenge. Four hours after challenge, the animals were euthanized, and skin tissue was assessed for edema and inflammation by measuring dye extravasation into the surrounding tissue. Punch biopsy of the injection sites (8 mm) were incubated in 2 ml of formamide at 80°C overnight. The concentration of the extravasated Evans blue dye was measured spectrophotometrically at OD610.

**CAIA.** Balb/c mice were passively sensitized by i.v. administration of collagen-induced arthritis monoclonal antibody blend (Chemicon International, Temecula, CA) on day 0, followed by LPS (25 μg) i.p. on day 2 (Terato et al., 1992; Kagari et al., 2003). Mouse IgG (i.v., day 0) and LPS (i.p., day 2) were administered to a separate group of mice as a negative control, as well as an untreated and nonimmunized naïve group. R406 was administered orally b.i.d for 14 days starting 4 h after antibody challenge on day 0. Vehicle (35% TPGS, 60% PEG 400, 5% propylene glycol) was given to control animals. For the assessment of clinical scores, see supplemental data.

**K/BxN Model.** Arthritis was induced in C57BL/6 mice by intraperitoneal injection of 150 μl of pooled sera from adult K/BxN mice on days 0 and 2. R406 or vehicle (35% TPGS, 60% PEG 400, and 5% propylene glycol) was administered orally 1 h before serum injection (Ji et al., 2002). R406 or vehicle was then administered orally b.i.d for 13 days. Ankle thickness and arthritis were scored daily. Scores of each limb (0, no disease; 1, subtle inflammation (metatarsal phalanges joints, individual phalanx, or localized edema); 2, easily identified swelling; and 3, swelling in all aspects of paw) were summed, with the maximal score of 12.

**Clinical Study.** This was a double blind, placebo-controlled, ascending single dose, randomized study in normal healthy human male volunteers conducted at a single clinical research unit. Informed consent statements were obtained from all subjects. Subjects were admitted to the unit 1 day before dosing and discharged 48 to 72 h after dosing. There were five groups of subjects with six to eight per group (one or two control subjects per group). The dose assignments were 80, 250, 400, 500, and 600 mg, respectively. The protocol was reviewed and approved by the Institutional Research Ethics Committee for the Drug Research Unit at Guy’s Hospital (London, UK). For detail on the study design, see supplemental data.

**Data Analysis.** All IC_{50} and EC_{50} values in biochemical and cell-based assays were determined using Prism 3.0 software and Prism Enzyme programs. Results are presented as the mean ± S.D. Statistical significance was determined using the two-tailed Student’s t test. Pharmacokinetic analyses were performed using a WinNonLin software package.

## Results

**R406 Specifically Blocks FcεRI Signaling in Mast Cells.** IgE-mediated activation of mast cells occurs through FcεRI receptors and results in degranulation and the synthesis of lipid mediators and cytokines (Galli et al., 2005). To find specific FcεRI signaling inhibitors, we screened for small molecule compounds that inhibited degranulation of CHMC elicited by FcεRI cross-linking with anti-IgE antibodies but not by the calcium ionophore ionomycin (Rossi et al., 2006). Medicinal chemistry of initial hits using cell-based structure-activity relationships resulted in the identification of R406. R406 dose-dependently inhibited anti-IgE-mediated CHMC degranulation measured as tryptase release (EC_{50} = 0.056 ± 0.02 μM) but showed no activity on ionomycin-triggered tryptase release, indicating that R406 is specific to FεR signaling and not degranulation per se (Fig. 1a). As intended, this specific inhibition also implies that R406 site of action is proximal to the receptor complex and upstream of calcium mobilization. Importantly, R406 also inhibited the anti-IgE-induced production and release of LTC_{4} and cytokines and chemokines, including TNFα, IL-8, and GM-CSF (Table 1).

**Syk Kinase Activity Is the Primary Target for R406.** Studies to date have shown that FcεRI signaling induced by receptor cross-linking is initiated by the sequential activation of the tyrosine kinases Lyn and Syk (Sriraganian, 2003; Galli et al., 2005). Lyn, a member of the Src kinase family, is the first kinase to be activated leading to the phosphorylation of FcεRy chain of the FcεRI complex on its ITAM motifs, the recruitment of Syk to the phosphorylated ITAM, and the subsequent phosphorylation of Syk on tyrosine 352 (Law et al., 1996). Activated Syk then directly phosphorylates the adaptor molecule LAT on tyrosine 191 (Galli et al., 2005).

### Table 1

R406 specifically inhibits Syk-dependent FcεR and BCR signaling

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Stimulation</th>
<th>Signaling Pathway</th>
<th>Readout</th>
<th>EC_{50}</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHMC</td>
<td>Anti-IgE</td>
<td>FcεRI</td>
<td>Degranulation</td>
<td>0.056</td>
<td>0.02</td>
</tr>
<tr>
<td>CHMC</td>
<td>Anti-IgE</td>
<td>FcεRI</td>
<td>Leukotriene LTC_{4}</td>
<td>0.093</td>
<td>0.076</td>
</tr>
<tr>
<td>CHMC</td>
<td>Anti-IgE</td>
<td>FcεRI</td>
<td>TNFα</td>
<td>0.118</td>
<td>0.031</td>
</tr>
<tr>
<td>CHMC</td>
<td>Anti-IgE</td>
<td>FcεRI</td>
<td>IL-8</td>
<td>0.140</td>
<td>0.016</td>
</tr>
<tr>
<td>CHMC</td>
<td>Anti-IgE</td>
<td>FcεRI</td>
<td>GM-CSF</td>
<td>0.158</td>
<td>0.025</td>
</tr>
<tr>
<td>CHMC</td>
<td>Anti-IgG</td>
<td>FcγRI</td>
<td>Degranulation</td>
<td>0.064</td>
<td>0.016</td>
</tr>
<tr>
<td>CHMC</td>
<td>Anti-IgG</td>
<td>FcγRI</td>
<td>Leukotriene LTC_{4}</td>
<td>0.041</td>
<td>0.08</td>
</tr>
<tr>
<td>CHMC</td>
<td>Anti-IgG</td>
<td>FcγRI</td>
<td>TNFα</td>
<td>0.082</td>
<td>0.053</td>
</tr>
<tr>
<td>CHMC</td>
<td>Anti-IgG</td>
<td>FcγRI</td>
<td>IL-8</td>
<td>0.053</td>
<td>0.012</td>
</tr>
<tr>
<td>CHMC</td>
<td>Anti-IgG</td>
<td>FcγRI</td>
<td>GM-CSF</td>
<td>0.056</td>
<td>0.014</td>
</tr>
<tr>
<td>Monocytic line, THP-1</td>
<td>Anti-IgG</td>
<td>FcγRI</td>
<td>TNFα</td>
<td>0.171</td>
<td>0.104</td>
</tr>
<tr>
<td>Primary human macrophages</td>
<td>Anti-IgG</td>
<td>FcγRI</td>
<td>TNFα</td>
<td>0.111</td>
<td>0.023</td>
</tr>
<tr>
<td>Primary human neutrophils</td>
<td>Anti-IgG</td>
<td>FcγRI</td>
<td>Oxidative burst</td>
<td>0.033</td>
<td>0.008</td>
</tr>
<tr>
<td>Primary human B-cells</td>
<td>Anti-IgM</td>
<td>BCR</td>
<td>CD68 up-regulation</td>
<td>0.048</td>
<td>0.026</td>
</tr>
</tbody>
</table>
critically contributing to the formation of signaling hubs necessary for downstream signal transduction.

Interestingly, R406 did not inhibit phosphorylation of Syk tyrosine 352 but inhibited the phosphorylation of LAT tyrosine 191, strongly suggesting that R406 inhibited Syk but not Lyn kinase activity (Fig. 1b). Consistent with the inability of inhibiting Lyn activity, R406 also did not block the Lyn-dependent phosphorylation of the FeRγ chain (Fig. 2). On the other hand, the Src family kinase inhibitor PP2 potently inhibited the Lyn-dependent phosphorylation of Syk tyrosine 352 and the subsequent Syk-dependent phosphorylation of LAT (Fig. 1b). Analogous results were obtained using murine bone marrow-derived mast cells, stimulated through their FcεRI and Ramos B cells, stimulated through their B-cell receptor, indicating that these results were not specific to CHMC (Fig. 2). Furthermore, inhibition of Syk by R406 resulted in inhibition of all phosphorylation events downstream of Syk signaling (Siraganian, 2003; Galli et al., 2005), including the phosphorylation of phospholipase Cγ1 (Tyr783), Akt/protein kinase B (Ser473), extracellular signal-regulated kinase (Thr202/Tyr204), p38 (Thr180/Tyr182), and c-Jun N-terminal kinase (Thr183/Tyr185) (Fig. 1c). Thus, the results show that R406 selectively inhibited Syk-dependent signaling in cells.

To confirm Syk inhibition by R406, we turned to biochemical Syk kinase studies. Fittingly, R406 potently inhibited Syk kinase activity in vitro with an IC50 of 41 nM, measured at an ATP concentration corresponding to its Km value. Moreover, subsequent enzyme kinetic studies showed R406 to be a competitive inhibitor for ATP binding with a Ki of 30 nM (Fig. 1d). In concordance with this mode of action, diffraction data collected from crystals of human Syk protein kinase domain (Ile358/Asn635) soaked with R406 indicated electron density consistent with R406 competing with ATP for binding to the same pocket (Fig. 1, f–h). Refined structures showed R406 binding the receptor in a “U”-shape conformation. The N1 on the pyrimidine and N2 on the linker secure the key contacts with the hinge region of Syk, whereas the methoxylated phenyl and heterocyclic ring enhance the interaction dramatically by multiple hydrogen bonds and hydrophobic interactions. A summary of crystallographic data and key interactions are shown in Supplemental Table 1. Taken together, the cellular and biochemical assays clearly demonstrate R406 as an ATP-competitive inhibitor of Syk kinase activity. In addition, a comparative analysis of over 1000 R406 analogs demonstrated a close positive correlation between inhibitory activity in IgE-mediated mast cell degranulation and in vitro Syk kinase activity, whereas no such correlation was observed with other tested kinase activities (data not shown).

For an initial selectivity assessment, R406 was profiled at 0.3 μM against a panel of over 90 in vitro kinase assays using a fixed ATP concentration of 10 μM for all kinase assays. Then, those significant kinases that were substantially inhibited by R406 were further tested in cells using phosphopeptide-specific antibodies for known phosphorylation targets of the respective kinases. The results indicated that, in cells, R406 inhibited all other kinases tested at 5- to 100-fold less potency than Syk as judged by phosphorylation of target proteins (Fig. 2). After Syk, Flt3 autophosphorylation was most potently inhibited by R406 at approximately 5-fold less potency than Syk activity (Fig. 2).

We also assessed selectivity of FcR signaling inhibition by R406 using a large panel of different cell-based counterassays representing a number of signaling pathways. The results, summarized in Table 2, indicate that R406 is more potent on Syk-dependent signaling than on Syk-independent pathways. Next to FcεRI signaling in CHMC, R406 most potently inhibited the signaling of IL-4 and IL-2 receptors (Table 2).

Finally, R406 was profiled on a broad commercial panel of receptor, ion channels, and unrelated enzymatic binding assays. The primary screening, performed in duplicate at 10 μM R406, resulted in three biochemical assays significantly inhibited (>50% inhibition). A subsequent semiquantitative concentration response on the three targets lead to estimated IC50 values: adenosine A3 receptor (IC50 = 0.081 μM), adenosine transporter (IC50 = 1.84 μM), and monoamine transporter (IC50 = 2.74 μM). Further testing in an assay measuring ligand-induced guanosine 5′-O-(thiotriphosphate) binding to the adenosine A3 receptor indicated that R406
exhibited antagonistic activity with an IC₅₀ estimated to be 0.093 μM. Because the addition of adenosine to CHMC did not result in activation of the cells (data not shown), we do not believe that this relatively potent inhibition of the adenosine A₃ receptor is contributing to the inhibitory effect of R406 on mast cells. The collective cellular and biochemical data indicate that R406 inhibits Syk kinase activity, but they also demonstrate some other inhibitory activities, which should be taken into account during the interpretation of toxicology and pharmacology studies.

**R406 Inhibits FcγR and B-Cell Receptor Syk-Dependent Signaling.** Having identified Syk kinase as a primary target for R406, we assessed its effect on other Syk-dependent pathways (Table 1; Fig. 3a) (Wong et al., 2004). Interestingly, in addition to FcεRI, CHMC also expressed FcγRI (CD64) on their cell surface; thus, we used these mast cells to test for FcγRI signaling. As expected, R406 also inhibited anti-IgG-induced CHMC degranulation (Fig. 3a) and production of LTC₄, TNFα, IL-8, and GM-CSF (Table 1). Similarly, R406 also potently inhibited TNFα production induced by FcγR-cross-linking in human macrophages derived from human monocytes or the monocytic cell line THP-1 (Table 1; Fig. 3a) (Turner et al., 2000). In contrast, roughly 10-fold higher levels of R406 were required to inhibit TNFα production induced by LPS, a mainly Syk-independent event (Table 1; Fig. 3a) (Turner et al., 2000). Likewise, in human neutrophils primed with TNFα, R406 inhibited oxidative burst induced by anti-IgG (Table 1; Fig. 3a) but not when induced by PMA (Table 2) (Asman et al., 1996). Thus, R406 specifically inhibited FcγR signaling in human mast cells, macrophages, and neutrophils.

**TABLE 2**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Stimulation</th>
<th>Receptor Type</th>
<th>Readout</th>
<th>EC₅₀</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHMC</td>
<td>Anti-IgE</td>
<td>ITAM (FcεRI)</td>
<td>Degranulation</td>
<td>0.056</td>
<td>0.02</td>
</tr>
<tr>
<td>Ramos B-cell line</td>
<td>IL-4</td>
<td>Cytokine (IL-4I)</td>
<td>CD23 up-regulation</td>
<td>0.192</td>
<td>0.01</td>
</tr>
<tr>
<td>Primary human T cells</td>
<td>IL-2</td>
<td>Cytokine (IL-2R)</td>
<td>Cell proliferation</td>
<td>0.190</td>
<td>0.07</td>
</tr>
<tr>
<td>Primary human T cells</td>
<td>αCD3/CD28</td>
<td>ITAM (TCR/CD28)</td>
<td>IL-2 production</td>
<td>0.448</td>
<td>0.09</td>
</tr>
<tr>
<td>A549 epithelial line</td>
<td>TNFα</td>
<td>Cytokine (TNFRI)</td>
<td>CD54 up-regulation</td>
<td>4.8</td>
<td>1.82</td>
</tr>
<tr>
<td>A549 epithelial line</td>
<td>IL-1</td>
<td>Cytokine (IL-1R)</td>
<td>CD54 up-regulation</td>
<td>7.87</td>
<td>3.76</td>
</tr>
<tr>
<td>A549 epithelial line</td>
<td>IFN-γ</td>
<td>Cytokine (IFNRI)</td>
<td>CD54 up-regulation</td>
<td>2.14</td>
<td>0.78</td>
</tr>
<tr>
<td>Jurkat T cell line</td>
<td>PMA</td>
<td>PKC</td>
<td>Oxidative burst</td>
<td>&gt;20</td>
<td></td>
</tr>
<tr>
<td>Primary human neutrophils</td>
<td>PMA</td>
<td>PKC</td>
<td>Degranulation</td>
<td>&gt;10</td>
<td></td>
</tr>
<tr>
<td>CHMC</td>
<td>Ionomycin</td>
<td>Calcium channels</td>
<td>TNFα</td>
<td>2.10</td>
<td>1.2</td>
</tr>
<tr>
<td>Monocytic line, THP-1</td>
<td>LPS</td>
<td>TLR-4</td>
<td>P-AKT (ICW)</td>
<td>1.19</td>
<td>0.28</td>
</tr>
<tr>
<td>Primary human macrophage</td>
<td>LPS</td>
<td>TLR-4</td>
<td>P-EGFR (ICW)</td>
<td>17.46</td>
<td>11.57</td>
</tr>
<tr>
<td>Huh7 hepatocyte line</td>
<td>Serum</td>
<td>General cell growth</td>
<td>Cell proliferation</td>
<td>15.1</td>
<td>7.48</td>
</tr>
<tr>
<td>A549 epithelial line</td>
<td>Serum</td>
<td>General cell growth</td>
<td>Cell proliferation</td>
<td>2.9</td>
<td>0.57</td>
</tr>
<tr>
<td>H1299 lung cancer line</td>
<td>Serum</td>
<td>General cell growth</td>
<td>Cell proliferation</td>
<td>6.3</td>
<td>3.5</td>
</tr>
<tr>
<td>HeLa</td>
<td>Insulin</td>
<td>Insulin receptor</td>
<td>P-AKT (ICW)</td>
<td>1.19</td>
<td>0.28</td>
</tr>
<tr>
<td>HeLa</td>
<td>EGF</td>
<td>EGF receptor</td>
<td>P-EGFR (ICW)</td>
<td>17.46</td>
<td>11.57</td>
</tr>
</tbody>
</table>

ICW, In-cell Western; EGF, epidermal growth factor; EGFR, EGF receptor; PKC, protein kinase C.
Syk activity also mediates signaling from the B-cell receptor (BCR), which consists of a membrane-anchored IgM antibody noncovalently associated with two ITAM-containing proteins, Ig-α and Ig-β (Pao et al., 1998; Turner et al., 2000). In primary B cells isolated from human peripheral blood, R406 strongly inhibited cell surface CD69 up-regulation induced by BCR-cross-linking with anti-IgM (Table 1; Fig. 3a). Hence consistent with being a Syk kinase inhibitor, R406 also blocked B-cell receptor signaling in B cells.

**R406 Effects on Macrophage, Neutrophil, and Platelet Function.** Because Syk is expressed in macrophages and neutrophils, we assessed R406 impact on general innate immune responses. For this reason, we tested R406 effects on leukocyte phagocytosis, oxidative burst, chemotaxis, and microbicidal activity. Notably, FcR signaling is but one pathway among many redundant systems used by monocytes, macrophages, and neutrophils to respond and eliminate microbes and foreign bodies (Janeway and Medzhitov, 2002). These other responses, which include Toll-like receptors (TLRs) and complement, are not regulated by Syk; consequently, R406 is not expected to significantly affect them.

Accordingly, R406 (up to 50 μM) had negligible effects on phagocytosis of FITC-labeled opsonized bacteria (Escherichia coli FITC) by monocytes and granulocytes in heparinized whole blood (Fig. 3b). Likewise, R406 did not significantly inhibit oxidative burst activity of human leukocytes induced by phagocytosis of opsonized E. coli (Fig. 3c), nor did R406 at 10 μM negatively affect granulocyte migration toward the chemotactic peptide N-formyl-Met-Leu-Phe (fMLP), whereas a 10 μM concentration of the positive control cytochalasin D inhibited leukocyte migration by >85%. Importantly, R406 (up to 20 μM) did not inhibit microbicidal activity by purified primary neutrophils as measured by the killing of opsonized S. aureus (Fig. 3d). Collectively, these results indicate that R406 does not appear to affect negatively on bacteria-induced innate immune responses.

Likewise, a multitude of redundant pathways regulate platelet activation and thrombus formation (Ruggeri, 2002). Because Syk kinase is known to play a role in platelet activation via the collagen receptor (glycoprotein VI) and some specific integrins (αIIbβ3) (Poole et al., 1997; Obergfell et al., 2002), we assessed the effect of R406 on platelet function. For this reason, we tested R406 effect on hemostasis in vivo by measuring the bleeding times after tail-tip amputation in...
mice. R406 dosed orally up to 100 mg/kg, resulting in systemic exposures up to 25 μM, did not extend bleeding time in mice, whereas aspirin at 100 mg/kg caused an 88% prolongation of bleeding time (Supplemental Table 2). Thus, high systemic exposures of R406 did not negatively affect bleeding times in mice.

**Effect of R406 in Mouse Models for IC-Mediated Inflammation.** To test R406 effects on IC-mediated inflammation, we first investigated the ability of R406 to inhibit the reverse passive Arthus reaction (Ravetch and Bolland, 2001). Prophylactic treatment of mice with R406 administered 1 h before immune complex challenge reduced the cutaneous reverse passive Arthus reaction by approximately 72 and 86% at 1 and 5 mg/kg, respectively, compared with the vehicle control. The net optical density reading of extravasated dye extracted after treatment with R406 at 1 or 5 mg/kg R406 was reduced from 0.14 (vehicle) to 0.04 or 0.02, respectively (p < 0.01). These findings correlate with the tissue area exhibiting dye extravasation and together show that R406 can inhibit local inflammatory injury mediated by ICs (Fig. 4, a and b).

Because activating Fcγ receptors expressed on synovial macrophages and mast cells are important in the onset of IC-mediated arthritis, we tested R406 in two mouse models of antibody-induced arthritis. In the passive anti-CAIA model (Terato et al., 1992; Kagari et al., 2003), treatment of the anticolonagen antibody-challenged mice with R406 reduced inflammation and swelling, and the arthritis progressed more slowly in treated animals than in vehicle controls (Fig. 4d). Starting on day 4, the majority of animals in the vehicle control group showed evidence of inflammation, predominantly in the hindpaws, and signs of progressive arthritis that reached a peak on day 7. The arthritis of hindpaws in R406-treated animals progressed slower than those of the control group and reached their disease peak on day 10.

Representative joint histopathology of study groups from mouse hindpaws at day 14 is shown in Fig. 4e. In vehicle-treated CAIA groups, the histopathological examination of both the right and left paws revealed a marked chronic synovitis, with thickened joints and synovial lining, pannus formation, and infiltration of neutrophils, lymphocytes, monocytes, and macrophages. In animals treated with R406, the damage of joints was markedly reduced, leaving them close to normal.

Similar results were obtained in the K/BxN serum transfer mouse model (Corr and Crain, 2002; Ji et al., 2002). Transfer of serum with affinity for glucose 6-phosphate isomerase from K/BxN mice into C57BL/6 mice results in the mast cell-dependent development of arthritis in the recipient mice. Treatment of injected C57BL/6 mice with 10 mg/kg R406 bid delayed the onset and reduced the severity of clinical arthritis. Paw thickening and clinical arthritis were reduced by approximately 50% (Fig. 4c).

Thus, R406 showed efficacy in the amelioration of the Arthus reaction and in reducing clinical symptoms in the CAIA and K/BxN models of RA. Taken together, IC-mediated inflammation was reduced by inhibition of Fc receptor signaling with R406.

**Effect of R406 on Basophil Activation and Platelet Aggregation in Healthy Human Volunteers.** To assess suitability for human studies, R406 was entered into a double blind, placebo-controlled, ascending single dose, randomized study in normal healthy male volunteers of ages ranging from 18 to 31 years and body mass index ranging from 20 to 28.

Pharmacokinetic assessment indicated that R406 was highly bioavailable. R406 plasma concentration increased in a dose-proportional fashion up to 400 mg and then reached a plateau (Fig. 5a). The maximal R406 concentration was gen-

![Fig. 5. Relationship between pharmacodynamic effect and plasma concentration of R406 in humans.](https://jpet.aspetjournals.org/)
erally reached between 1.2 and 1.3 h after dosing, and the half-life was approximately 15 h (data not shown).

To test the effects of R406 on basophil activation, heparinized blood from volunteers was stimulated ex vivo with anti-IgE, and degranulation was measured as CD63 cell-surface up-regulation on basophils by flow cytometry (Knol et al., 1991; Ebo et al., 2002). CD63, localized on granule membranes of resting basophils, gets exposed to the cell surface upon degranulation, and thus can be labeled with anti-CD63 antibodies. R406 significantly inhibited CD63 cell-surface expression in a dose-dependent manner (Fig. 5a). The magnitude and duration of the effect increased as R406 plasma concentration increased, and at the higher doses, there was significant inhibition of CD63 expression for as long as 24 h after dosing (Fig. 5b). The degree of inhibition was highly correlated with plasma concentration of R406 (Fig. 5c). The plasma concentration that produces a 50% reduction of basophil activation was 496 ± 42 ng/ml, which corresponds to an EC_{50} of 1.06 μM.

We also tested the effect of R406 on platelet aggregation in platelet-rich plasma prepared from the dosed volunteers. Even at the highest doses, there was no inhibition of collagen or ADP-induced platelet aggregation (Supplemental Table 3).

Finally, safety assessment indicated no effects on any hematologic or chemistry safety parameters. Subjects dosed at 600 mg complained of postural dizziness (or in one case dizziness) more frequently than those dosed with vehicle or lower doses (5/6 versus 1/8 and 4/21, respectively). Peripheral blood mononuclear flow cytometry showed no significant dose or time-related effect on the circulating subsets of mononuclear cells or on a marker of monocyte activation (as determined by mean fluorescence intensity of CD86 on CD14 cells). However, on subsequent studies, R406 treatment tended to cause a dose-dependent reduction of circulating CD45^+CD14^+ mononuclear cells 4 h after a single dose, which was reversed when measured 20 h later (data not shown). Because this reduction occurred at doses resulting in R406 exposures well over the pharmacodynamic EC_{50}, as measured by basophil degranulation, it is unclear whether Syk inhibition contributed to this effect.

**Discussion**

Several lines of evidence have brought to the forefront the importance of ICs, activating Fc receptor signaling, and, by extension, Syk in the inflammatory response in many autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis in addition to immune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis in addition to asthma (Takai, 2002; Wong et al., 2004; Firestein and Corr, 2005).

Using cell-based structure activity relationships with human mast cells, we identified R406 as a specific FceRI signaling inhibitor (Fig. 1a). Here we report the initial characterization of R406 and show its potential for modulating Syk activity in human disease. We determined the R406 primary mode of action to be the inhibition of Syk kinase activity in cells (Fig. 1c) in an ATP-competitive manner (Fig. 1, e–i).

Our experience shows that isolated biochemical assays often fail to faithfully mimic the “native state” kinase activity inside cells. Thus, to assess selectivity, our preferred approach is to examine kinase inhibition in their cellular context. Therefore, we followed an initial profiling of R406 on a panel of kinase assays with careful assessments using antiphosphopeptide Western blots (Fig. 2). In addition, we complemented this assessment using cell-based assays representing different signaling systems that are regulated by multiple kinases (Table 2).

In this way, we found that R406 potently inhibited all Syk-dependent cell-based assays tested, which included activating Fc receptor signaling in human macrophages, neutrophils and mast cells, and B-cell receptor signaling in human B cells (Table 1). R406 was relatively selective to Syk inhibition, as assessed by inhibition of phosphorylation in cells, and with a large panel of off-target Syk-independent cell-based assays (Fig. 2; Table 2). Beyond Syk, R406 inhibited next in potency assays dependent on Flt3, Jak, and Lck (Fig. 2; Table 2), which for inflammatory processes might be considered to be desirable properties (Firestein, 2003; Smolen and Steiner, 2003). Thus, altogether the results suggest a favorable cellular profile for R406 as an anti-inflammatory agent that primarily inhibits Syk kinase activity.

The Syk expression in macrophages and neutrophils prompted us to examine whether Syk inhibition by R406 interfered with innate immune responses. Previous reports with murine Syk-deficient cells had suggested that redundant systems allow macrophages and neutrophils to respond to bacterial pathogens in the absence of Syk (Turner et al., 2000; Mocsai et al., 2003). Indeed, neutrophil/macrophage phagocytosis, chemotaxis, oxidative burst, and microbialic responses to bacterial products or opsonized bacteria were without significant effect at high micromolar concentrations of R406 (Fig. 3).

Likewise, Syk kinase is also known to play a role in platelet activation via the collagen receptor (glycoprotein VI) and specific integrins (αIIbβ3) (Clark et al., 1994; Poole et al., 1997). However, Law et al. (1999) reported normal bleeding times in mice reconstituted with Syk-deficient platelets and, notably, that humans deficient in glycoprotein VI exhibited only mild bleeding tendency and otherwise normal coagulation parameters (Moroi et al., 1989). Consistent with these results, R406 dosed orally in mice had no effect on bleeding times (Supplemental Table 2). Moreover, no inhibition of platelet aggregation induced by collagen or ADP in platelet-rich plasma from the human volunteers dosed with R406 was observed (Supplemental Table 3). Thus, taken together, R406 faithfully phenocopies Syk deficiency without undue deleterious effects on hemostasis or innate immune responses to pathogens.

Next, we sought to assess the effects of Syk inhibition using R406 in several animal models of IC-mediated inflammatory processes known to be dependent on the activating Fcγ receptors (Hazenbos et al., 1996; Sylvestre and Ravetch, 1996; Corr and Crain, 2002; Kagari et al., 2003). Oral administration of R406 dose-dependently inhibited edema formation and hemorrhage in the reverse passive Arthus reaction (Fig. 4, a and b). This is probably the result of R406 inhibition of IC-induced degranulation and mediator release by perivascular mast cells, as well as inhibition of macrophage and Langerhans cell activation. The complete inhibition by R406 mimicked results observed with Fcγ-deficient mice (Hazenbos et al., 1996; Sylvestre and Ravetch, 1996), implying that Syk kinase activity mediates most, if not all, of the FcR-dependent inflammatory response in the Arthus reaction.
Likewise, R406 treatment ameliorated joint swelling induced in mice with arthritogenic anti-collagen type II antibodies or K/BxN serum with affinity for glucose 6-phosphate isomerase (Fig. 4, d–e). The reduction of paw inflammation caused by R406 was not as complete as that observed in FcRγ-deficient mice (Kagari et al., 2003). This is probably due to insufficient drug systemic exposure as R406 is rapidly metabolized in mice (Supplemental Fig. 1) and thus unlikely to reproduce the totality of the lesion in the knockout model. Significantly, despite some swelling, joint histopathology assessment indicated that R406 treatments resulted in marked reduction of synovitis, pannus formation, and leukocyte infiltration (Fig. 4e). It is possible that the diverse beneficial effects of inhibition with R406 are due to its broad effects on the many cells populating the inflamed synovium. Syk plays a central role in IC-mediated activation of sentinel cells (mast cells and macrophages), leading to vascular leakage, cytokine production, and leukocyte infiltration; in activation of dendritic cells needed for disease propagation; and in activation of effector cells (neutrophils and macrophages) and osteoclast differentiation, resulting in tissue damage and bone destruction (Wong et al., 2004).

In this regard, we have recently shown that R406 treatment has significant activity in a rodent collagen-induced arthritis model, with improvement in clinical scores, histopathology, and joint radiography (Brahn et al., 2004). In rat models of RA, treatment with R406 lead to inhibition of inflammation as measured by reduction of cytokines in the synovial fluid and cartilage oligomeric matrix protein in the serum. Moreover, influx of inflammatory cells into the synovium was inhibited and bone erosion was drastically reduced (P. Pine, B. Chang, S. Wang, A. Lau, J. Lee, M. Banguiero, D. Payan, E. Grossbard, and E. Brahn, manuscript in preparation).

Likewise, we have recently shown in murine asthma models that R406 ameliorates airway hyperresponsiveness, pulmonary eosinophilia, and goblet cell hyperplasia (Matsubara et al., 2005, 2006). IgE-FcεRI signaling is intimately associated with allergic conditions, and pharmacological Syk inhibition has previously been shown to result in the reduction of allergic airway inflammation (Yamamoto et al., 2003; Wong et al., 2004). Notably, a Syk inhibitor delivered via nasal spray was shown to have a clinically significant effect on the symptoms of seasonal allergic rhinitis in a park environment (Meltzer et al., 2005). Thus, R406 has therapeutic potential for both allergic and autoimmune inflammatory disorders. The inhibition of Syk kinase and Fc receptor signaling is probably a primary mechanism for these effects; however, the impact of additional beneficial effects by inhibition of Lck, Jak, and Fli3 activities is not to be ignored and remains to be investigated further. Likewise, the potential inhibition of the adenosine A3 receptor by R406 needs to be considered, although the physiological significance of pharmacological modulation of human adenosine A3 receptors remains unclear.

Interestingly, Syk kinase has been reported to play a role in TNFα signaling in selected cell types intimately involved in inflammatory processes. Indeed, Cha et al. (2006) have recently demonstrated the presence of activated Syk in the intimal lining of synovial tissue from rheumatoid arthritis patients. Moreover, they showed that R406 inhibited cytokine and matrix metalloproteinase production from TNFα-induced RA fibroblast-like synoviocytes.

Finally, antibody and IC-mediated inflammatory diseases usually develop in two distinct but overlapping phases: an immune response to antigens and the subsequent inflammatory process (Smolen and Steiner, 2003; Firestein and Corr, 2005). Here we have shown the salutary effects of R406 on inflammation. Syk kinase is also essential for BCR signaling in B cells and FcR signaling in dendritic cells (Turner et al., 2000; Sedlik et al., 2003). Thus, it will be interesting to assess in the future R406 effects on humoral immune responses, including antibody production and immunoglobulin class switching.

Importantly, R406 proved to be highly bioavailable in human subjects (Fig. 5a). Oral administration of R406 induced a dose-dependent inhibition of CD63 cell-surface expression in peripheral blood basophils following stimulation by anti-IgE. The magnitude of the inhibitory effect and its duration highly correlated with the plasma concentration of R406. The disparity between the cell-based EC50 and the in vivo EC50 may be attributable to high-protein binding of R406 in human plasma (>98%). We have seen a comparable shift in EC50 in Syk-dependent and Syk-independent cell-based assays when human serum was added (data not shown). Notably, no remarkable adverse effects were observed at levels of R406 that inhibited basophil activation. Overall, the pharmacokinetic and pharmacodynamic data suggest that twice per day, or even once per day, dosing might be sufficient to achieve the desired biologic reduction of Syk activity in target tissues.

In summary, we have developed R406 as a potent Syk kinase inhibitor using biology-directed drug discovery. We have shown that R406 has potential for broad anti-inflammatory properties because it inhibits several critical nodes of the inflammatory cascade without interfering markedly with the innate immune response or hemostasis. Our results with R406 support Syk inhibition as a novel therapeutic approach for inflammatory arthritis and other immune-mediated inflammatory diseases.

Acknowledgments

We thank Peter Chu, Stacey Huyhn, Weidunn Xu, John McLaughlin, Wayne Lang, Hong Ren, Kathryn Hjerrild, and Pam Witte for expert technical support. We also thank Diane Mathis for the K/BxN model. We are especially grateful to Theresa Musser, Michael Sterba, and David Lau for advice, support, and enthusiasm throughout the project.

References


Address correspondence to: Dr. Esteban S. Masuda, Rigel Pharmaceuticals, 1180 Veterans Blvd, South San Francisco, CA 94080. E-mail: emasuda@rigel.com
Correction to R406, an Orally Available Spleen Tyrosine Kinase Inhibitor Blocks Fc Receptor Signaling and Reduces Immune Complex-Mediated Inflammation

In the above article [Braselmann S, Taylor V, Zhao H, Wang S, Sylvain C, Baluom M, Qu K, Herlaar E, Lau A, Young C, Wong BR, Lovell S, Sun T, Park G, Argade A, Jurcevic S, Pine P, Singh R, Grossbard EB, Payan DG, and Masuda ES (2006) *J Pharmacol Exp Ther* 319:998-1008], the Western Blot of phosphorylated Stat6 in Ramos B-cells stimulated with interleukin 4 (IL4) was accidentally misaligned during the preparation of Fig. 2. The corrected figure appears below.

The online versions will be corrected.

The authors regret this error and any inconvenience it may have caused.
### Syk target
- **BMMCC αIgE**
  - DMSO: P- LAT (Y191) Loading Control
  - R406: P- LAT (Y191) Loading Control

- **Ramos B-cell αIgM**
  - DMSO: P- BLNK (Y96) Loading Control
  - R406: P- BLNK (Y96) Loading Control

### Flt3 target
- **MV4-11 constitutive**
  - DMSO: P- Flt3 (4G10) IP Control: Flt3
  - R406: P- Flt3 (4G10) IP Control: Flt3

### c-Kit target
- **BMMCC SCF**
  - DMSO: P- cKit (Y719) Loading Control
  - R406: P- cKit (Y719) Loading Control

### Lck target
- **Primary T-cell αCD3/CD28**
  - DMSO: P- Zap70 (Y319) Loading Control
  - R406: P- Zap70 (Y319) Loading Control

### Jak 1 + 3 target
- **Ramos B-cell IL4**
  - DMSO: P- Stat6 (Y641) Loading Control
  - R406: P- Stat6 (Y641) Loading Control

### Lyn target
- **CHMC αIgE**
  - DMSO: P- FcRγ (4G10) IP Control: FcRγ
  - R406: P- FcRγ (4G10) IP Control: FcRγ

- **BMMCC αIgE**
  - DMSO: P- Syk (Y352) Loading Control
  - R406: P- Syk (Y352) Loading Control