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Preclinical Characterization of GLPG0634, a Selective Inhibitor of JAK1, for the Treatment of Inflammatory Diseases

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The JAKs receive continued interest as therapeutic targets for autoimmune, inflammatory, and oncological diseases. JAKs play critical roles in the development and biology of the hematopoietic system, as evidenced by mouse and human genetics. JAK1 is critical for the signal transduction of many type I and type II inflammatory cytokine receptors. In a search for JAK small molecule inhibitors, GLPG0634 was identified as a lead compound belonging to a novel class of JAK inhibitors. It displayed a JAK1/JAK2 inhibitor profile in biochemical assays, but subsequent studies in cellular and whole blood assays revealed a selectivity of ~30-fold for JAK1-over JAK2-dependent signaling. GLPG0634 dose-dependently inhibited Th1 and Th2 differentiation and to a lesser extent the differentiation of Th17 cells in vitro. GLPG0634 was well exposed in rodents upon oral dosing, and exposure levels correlated with repression of Mx2 expression in leukocytes. Oral dosing of GLPG0634 in a therapeutic set-up in a collagen-induced arthritis model in rodents resulted in a significant dose-dependent reduction of the disease progression. Paw swelling, bone and cartilage degradation, and levels of inflammatory cytokines were reduced by GLPG0634 treatment. Efficacy of GLPG0634 in the collagen-induced arthritis models was comparable to the results obtained with etanercept. In conclusion, the JAK1 selective inhibitor GLPG0634 is a promising novel therapeutic with potential for oral treatment of rheumatoid arthritis and possibly other immune-inflammatory diseases. The Journal of Immunology, 2013, 191: 3568–3577.

The online version of this article contains supplemental material.

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The Journal of Immunology

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he JAKs are cytoplasmic tyrosine kinases critical for intracellular signal transduction of many cytokines, growth factors, and hormones. Four human JAKs have been described: JAK1, JAK2, JAK3, and TYK2. JAKs bind to the intracellular moieties of type I and type II receptors, and JAK homo- or heterodimers become activated upon ligand binding. The JAKs phosphorylate each other followed by phosphorylation of tyrosine residues on the intracellular domains of the receptors. These phosphorylated residues serve as docking sites for STAT transcription factors. JAK phosphorylation of the STAT proteins results in their nuclear translocation and provides transcriptional output for the cytokine ligands (1, 2). JAKs play important roles in the functioning of the immune system. Mouse and human genetics studies linked deficiencies of JAK1 and JAK3 to severe combined immune deficiency and TYK2 to increased susceptibility to infections (3, 4). JAK2 serves signal transduction for inflammatory cytokines such as IFN-γ, IL-12, IL-23, and GM-CSF (2, 5). Hence, JAKs have been targeted for their therapeutic potential in immune-inflammatory disorders. In fact, small-molecule JAK inhibitors proved efficacious in a range of animal disease models and have already shown promise in the clinic for organ transplant rejection, rheumatoid arthritis (RA), psoriasis, dry eye disease, myelofibrosis, inflammatory bowel disease, and asthma (5–10). JAK2-mediated side effects observed in clinical trials such as anemia, neutropenia, and thrombocytopenia appear to be the main cause for not fully exploiting the pharmacodynamic potential of these JAK inhibitors (11–13). Recent findings suggest that JAK1 dominates JAK1/JAK3/γ chain signaling, suggesting that JAK1 inhibition might be largely responsible for the in vivo efficacy of JAK inhibitors in immune-inflammatory diseases (14–16). These results indicate that a selective JAK1 inhibitor could provide an increased therapeutic window allowing for higher dosing and efficacy while avoiding dose-limited pharmacology as observed for the pan-JAK inhibitors.

To exploit the therapeutic potential of JAK1 for the treatment of immune-inflammatory diseases, we set out to identify selective JAK1 inhibitors. One of the lead compounds, GLPG0634, was shown to selectively inhibit JAK1-dependent signaling in cellular and whole blood assays (WBAs) and showed remarkable efficacy in collagen-induced arthritis (CIA) disease models for RA in both mouse and rat.

Materials and Methods

Small-molecule kinase inhibitors

Focused kinase collections were sourced from BioFocus (Essex, U.K.). GLPG0634 was synthesized by Galapagos medicinal chemists. Tofacitinib and baricitinib were sourced from Shanghai Haoyuan Chemexpress (Shanghai, China) and Charnwood Molecular (Loughborough, U.K.), respectively.
Biochemical assays

IC₅₀ determination. Recombinant JAK1, TYK2 (Invitrogen), JAK2, and JAK3 (Carna Biosciences) were used to develop activity assays in 50 mM HEPES (pH 7.5), 1 mM EDTA, 10 mM MgCl₂, 2 mM DTT, and 0.01% Tween 20. The amount of JAK protein was determined per aliquot, maintaining initial velocity and linearity over time. The ATP concentration was equivalent to 4 x the experimental Kₘ value and the substrate concentration (ULight-conjugated JAK-1(Tyr1022) peptide; PerkinElmer) corresponded to the experimentally determined Kₘ value. After 90 min incubation at room temperature (RT), the amount of phosphorylated substrate was measured by addition of 2 nM europium-anti-phosphotyrosine Ab (PerkinElmer) and 10 mM EDTA in Laemmli buffer (PerkinElmer). Compound IC₅₀ values were determined by preincubating the enzyme with compound at RT for 60 min, prior to the addition of ATP.

Kₘ determination. Dissociation constants were determined at Proteos Biostuctures (Martinsried, Germany). Proprietary fluorescently labeled ATP mimetics with fast dissociation rates (PRO13, PRO14, and PRO15 for JAK1, JAK2, and JAK3, respectively) were incubated with JH1 domains of purified JAKs in 20 mM MOPS (pH 7.5), 1 mM DTT, 0.01% Tween 20, and 500 mM hydroxyctein (JAK3 only) for 30 min. Compounds (concentrations ranging from 520 pM to 1.1 μM) were added in 100% DMSO and time dependency of reporter displacement was measured. IC₅₀ values corresponding to 50% probe displacement were obtained and Ka values were calculated according to the Cheng–Prusoff equation.

Cellular assays

STAT6 phosphorylation induced by IL-4. THP-1 cells (ATCC TIB-202) were preincubated with compound at RT for 1 h, incubated with IL-4 (10 ng/ml) for 30 min, and processed for AlphaScreen analysis (Biostructures) (17). For Th2 cell polarization, cells were cultured in the presence of 10 ng/ml anti-IL-4 Ab (MAB204; R&D Systems), 10 ng/ml IL-2 (R&D Systems), and 10 ng/ml IL-12 (R&D Systems) (17). For Th2 cell polarization, cells were cultured in the presence of 10 ng/ml anti-IFN-γ Ab (Becton Dickinson), 25 ng/ml IL-4 (R&D Systems), and 10 ng/ml IL-2 (17). For Th17 cell polarization, a mixture of the following cytokines was used: 10 ng/ml IL-6 (R&D Systems), 10 ng/ml IL-1β (R&D Systems), 1 ng/ml TGF-β (PeproTech), and 100 ng/ml IL-23 (R&D Systems) (18). To monitor effects of compounds on Th cell differentiation, compounds were added at indicated concentrations at the start of T cell differentiation. After 5 d, RNA was extracted using an RNeasy Mini kit (Qiagen), reverse transcribed, and the extent of Th subset differentiation was monitored by determining expression of IFN-γ (Th1 marker), IL-13 (Th2 marker), or IL-17 (Th17 marker) using real-time PCR on the Viia7 thermocycler with predesigned TaqMan Assay-on-Demand gene expression primer/probe sets (Applied Biosystems). Gene expression was normalized to 18S and expressed as ΔCt values, with ΔCt = Ctgene – Ct18S or expressed as relative mRNA level of specific gene expression as obtained using the 2-ΔCt method.

Human WBAs

Human blood was collected from healthy volunteers, who gave informed consent, into sodium heparin vacutainer tubes by venipuncture. After incubation with compounds at 37°C for 30 min, blood was triggered with either recombinant human IL-6 (10 ng/ml; R&D Systems), recombinant human IL-2 (2 ng/ml; R&D Systems), universal INF-α (1000 U/ml; PBL Biomedical Laboratories), recombinant human GM-CSF (20 pg/ml; PeproTech), or vehicle (PBS plus 0.1% [w/v] BSA) at 37°C for 20 min and treated with 5% DMSO for 10 min. After washing, cells were fixed in Cytofix/Cytoperm (BD Biosciences) buffer and permeabilized with Phosflow perm buffer III (BD Biosciences) on ice for 30 min. After blocking (Fc blocking reagent; Miltenyi Biotec), pSTAT6 was detected with mouse anti-human PE-labeled anti-pSTAT6 Ab (BD Biosciences).

Knockdown experiments. HeLa and HCT116 cells obtained from the American Type Culture Collection were transfected with 50 nM ON-TARGETplus SMARTpool small interfering RNA (siRNA) for human JAK1, JAK2, JAK3, or TYK2, or with nontargeting or GAPDHnegative control siRNAs (Dharmacon) using Lipofectamine RNAiMAX transfection reagent from Invitrogen. Four days after transfection cells were starved overnight and stimulated with IL-6/IL-6R (both 250 ng/ml) for 20 min and pSTAT1 levels were determined using AlphaScreen technology (PerkinElmer) according to the manufacturer’s protocol.

T cell differentiation studies. PBMCs were isolated from buffy coats of healthy donors (Blood Transfusion Center, Red Cross, Leuven, Belgium) using density gradient centrifugation on Lymphoprep. Naïve CD4+ T cells were further isolated by depletion of non–T helper and memory CD4+ T cells using a naïve CD4+ T cell isolation kit II (Miltenyi Biotec). Naïve naïve CD4+ T cells were stimulated with plate-bound anti-CD3 (3 μg/ml; R&D Systems), 100 ng/ml IL-2 (R&D Systems), and 10 ng/ml IL-12 (R&D Systems) (17). For Th2 cell polarization, cells were cultured in the presence of 10 ng/ml anti-IL-4 Ab (MAB204; R&D Systems), 10 ng/ml IL-2 (R&D Systems), and 10 ng/ml IL-12 (R&D Systems) (17). For Th17 cell polarization, a mixture of the following cytokines was used: 10 ng/ml IL-2 (R&D Systems), 10 ng/ml IL-1β (R&D Systems), 1 ng/ml TGF-β (PeproTech), and 100 ng/ml IL-23 (R&D Systems) (18). To monitor effects of compounds on T cell differentiation, compounds were added at indicated concentrations at the start of T cell differentiation. After 5 d, RNA was extracted using an RNeasy Mini kit (Qiagen), reverse transcribed, and the extent of Th subset differentiation was monitored by determining expression of IFN-γ (Th1 marker), IL-13 (Th2 marker), or IL-17 (Th17 marker) using real-time PCR on the Viia7 thermocycler with predesigned TaqMan Assay-on-Demand gene expression primer/probe sets (Applied Biosystems). Gene expression was normalized to 18S and expressed as ΔCt values, with ΔCt = Ctgene – Ct18S or expressed as relative mRNA level of specific gene expression as obtained using the 2-ΔCt method.

Pharmacokinetics

Formulations. GLPG0634 was formulated in polyethylene glycol 200/0.9% NaCl (60/40; v/v) for i.v. administration and in 0.5% (v/v) methylcellulose for oral administration for all in vivo studies described. Compound purity was >95% as measured by HPLC.

Animals. Male Sprague Dawley rats (180–200 g) and CD1 mice (23–25 g) were obtained from Janvier and Harlan (France), respectively. Two days before administration of compound, rats underwent surgery to place a catheter into the jugular vein under isoflurane anesthesia. Animals were deprived of food for at least 16 h before oral dosing until 4–6 h after. Before oral dosing, animals were deprived of food for at least 12 h before compound administration until 4 h after administration. All in vivo experiments were carried out in a dedicated pathogen-free facility (22°C). Animal care was in accordance with the French guidelines about the use of animals in scientific research. All procedures involving animals (i.e., housing and care, method of euthanasia, and experimental protocols) were conducted in accordance with a code of practice established by the local ethical committee (Galapagos).

Pharmacokinetic studies. GLPG0634 was orally dosed as a single esophageal gavage at 5 mg/kg (dosing volume of 5 ml/kg) and i.v. dosed as a bolus via the caudal vein at 1 mg/kg (dosing volume of 5 ml/kg). In the rat study, each group consisted of three rats and blood samples were collected via the jugular vein. In the mouse study, each group consisted of 21 mice (n = 3/time point) and blood samples were collected by intracardiac puncture under...
isolation. Male DBA/1J mice (male, 6 wk old) were obtained from Janvier (Laval, France).

MATERIALS. CFA and IFA were purchased from Difco (Detroit, MI). Bovine collagen type II (CII) was obtained from Chondrex (Redmond, WA). All other reagents used were of reagent grade and all solvents were of analytical grade.

CIA. One day before the start of the experiment, CII solution (2 mg/ml) was prepared with 0.05 M acetic acid and stored at 4°C. Just before the immunization, equal volumes of IFA and CII were mixed by a homogenizer in a precooled glass bottle in an ice water bath. For rat CIA experiments, the emulsion (0.2 ml) was injected intraadermally at the base of the tail at day 1 and again at day 8. This immunization method was modified from published methods (19). The in vivo efficacy of GLPG0634 was determined after daily oral administration for a period of 14 d after onset of disease (average clinical score at onset, 2.5 ± 0.3; 10 rats/treatment group) over the dose range 50 mg/kg to 1 mg/kg. The oral enantiomer etanercept (Wyeth Pharmaceuticals, Taplow, U.K.) was administered three times per week at 10 mg/kg by i.p. injection. A fully active dose was reported to require repeated dosing in the 3–9 mg/kg range (20). In our model of Dark Agouti female rats, disease normalization was reached for 10 mg/kg enantiomer dosed three times a week as measured by clinical score, inflammation, bone resorption, pannus, and cartilage damage. At day 7 or 11, 200 μl blood was collected by retro-orbital puncture with lithium heparin as anticoagulant at predose and 1, 3, and 6 (n = 2 or 3/time point) for steady-state pharmacokinetics analysis. At sacrifice, hind paws were removed for gene expression analysis in mouse paws. Hind paws were dissected by cutting above the ankle joint and removing the digits. The remaining tissue was divided in two tubes and either left untreated (control) or treated with 520 U/ml rat IFN-α at 37°C for 1 h. RBCs were lysed (buffer EL; Qiagen) and the WBCs pelleted, dissolved, and homogenized in 350 μl buffer RLIT (Qiagen). Total RNA was extracted using the QiAamp RNA Blood Mini kit (Qiagen) and 500 ng was reverse transcribed using TaqMan RT kit (Applied Biosystems) with oligo(dT) priming. Five microarrays of 5× diluted cDNA preparations were used for real-time quantitative PCR (TaqMan technology, using a StepOnePlus thermocycler, Applied Biosystems) with gene-specific probes and primers designed according to standard procedures.

For the analysis of gene expression in mouse whole blood cells (circulating leukocytes), blood was sampled in RNAprotect tubes (Qiagen) and processed using the RNeasy protect animal blood kit (Qiagen). Total RNA (300 ng) was reverse transcribed using a high-capacity cDNA synthesis kit (Applied Biosystems) with random hexamers. Quantitative PCR reactions were performed using QuantFast SYBR Green PCR Master mix (Qiagen) and gene-specific primer pairs for β-actin (Eurogentec) and QuantTect primer assays for all other genes analyzed (Qiagen). Reactions were carried out with a denaturation step at 95°C for 5 min followed by 40 cycles (95°C for 10 s, 60°C for 1 min) in a Viia7 real-time PCR system (Applied Biosystems). Real-time PCR data for each target gene was expressed as ΔCt, corresponding to Ct obtained for the gene of interest normalized with the Ct of the β-actin gene. Gene expression analysis in mouse paws. Hind paws were dissected by cutting above the ankle joint and removing the digits. The remaining tissue was transferred into 2 ml homogenization tubes (Quality Scientific Plastics) containing 1 mm zirconium beads (BioSpec Products) and 750 μl Trizol reagent (Invitrogen). Tissue samples were homogenized using the Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) programmed to shake samples three times for 30 s at 5300 rpm with 5-s pauses between the homogenization steps. Total RNA was isolated as recommended by the Trizol supplier and further purified with Nucleospin RNA kit (Macherey-Nagel) according to the manufacturer’s instructions. RNA yields were determined by spectrophotometry at 260/280 nm. Reverse transcription of RNA and quantitative PCR were performed as described above. For statistical analysis, a two-way ANOVA followed by a Dunnett post hoc test versus the CIA-vehicle group was performed. Analyte measurement in mouse sera. Quantification of analytes in mouse sera was performed at Myriad RBM using mouse CytokineMAP A v1.0, mouse CytokineMAP B v1.0, and mouse CytokineMAP C v1.0.

Results.

To identify new JAK1 inhibitors, a kinase-focused collection of ~10,000 small molecules was screened in a JAK1 biochemical assay. From this screen a triazolopyridine series was identified as a tractable hit series. Establishment of a detailed structure-activity relationship in this series led to the identification of GLPG0634 as one of the lead compounds of the series. Characterization of GLPG0634 at the biochemical level indicated a selective inhibition of JAK1 and JAK2 over JAK3 and TYK2 with a rank order of potency of JAK1 > JAK2 > TYK2 > JAK3 (Table I). IC50 values determined in tyrosine kinase inhibition assays correlated with Kd values determined in ligand displacement assays (Table I).

Several cellular assay set-ups were applied to elucidate the potency and the JAK selectivity profile in a cellular environment. Cell lines were preincubated with GLPG0634 and treated with cytokines that employ different JAK heterodimeric or JAK2 homodimeric complexes for signaling. GLPG0634 inhibited IL-2- and IL-4–induced JAK1/JAK3/tyk2 signaling and IFN-γ–induced JAK1/tyk2 type II receptor signaling most potently. IC50 values ranged from 150 to 760 nM (Table I). IFN-γ– and OSM–induced JAK1/JAK2 signaling mediated by type II and gp130 receptor complexes and IL-3–induced JAK2/β2, signaling were inhibited with low
micromolar potencies (Table I). JAK2 homodimer–mediated signaling induced by EPO or PRL was inhibited with the lowest potency. IC_{50} values could not be determined accurately but were >10 μM. Inhibition of JAK3 and TYK2 is unlikely to contribute to GLPG0634 inhibition of JAK/STAT signaling in view of the potency difference measured in biochemical assays between JAK1 and JAK2 versus JAK3 and TYK2 (Table II). Hence, GLPG0634 preferentially inhibits JAK/STAT signaling involving JAK1 than JAK2 kinase in a cellular context. To test the relative contributions of JAK1 versus JAK2 in a physiological relevant model, GLPG0634 was tested for inhibition of cytokine-induced STAT phosphorylation in human WBAs. There was a particular interest in comparing GM-CSF/STAT5 versus IL-6/STAT1 signaling in myeloid and T cells, respectively. GM-CSF relies on a JAK2 homodimer for intracellular signaling, and IL-6–mediated phosphorylation of STAT1 is JAK1–dependent, as shown for T cells by Ghoreschi et al. (22). Similar results were obtained by applying an siRNA knockdown approach in HeLa and HCT116 cells. Transfection with siRNAs targeting the individual JAK family members resulted in a knockdown of the individual JAK family member. The IC_{50} values corresponding to 50% probe displacement were obtained and K_{d} values calculated according to the Cheng-Prusoff equation.

ND, Not determined

**Table I. Potency and selectivity of GLPG0634 in JAK biochemical assays**

<table>
<thead>
<tr>
<th>Recombinant Human Kinase</th>
<th>IC_{50} (nM, ± SEM; n = 2–4)</th>
<th>K_{d} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAK1</td>
<td>10 ± 0.8</td>
<td>11</td>
</tr>
<tr>
<td>JAK2</td>
<td>26 ± 5.4</td>
<td>32</td>
</tr>
<tr>
<td>JAK3</td>
<td>810 ± 180</td>
<td>300</td>
</tr>
<tr>
<td>TYK2</td>
<td>116 ± 39</td>
<td>ND</td>
</tr>
</tbody>
</table>

IC_{50} values for inhibition of recombinant JAK1, JAK2, JAK3, and TYK2 by GLPG0634 were determined by measuring the incorporation of phosphate into an ULight-JAK-1(Tyr1023) peptide using an europium-labeled anti-phosphotyrosine Ab. The K_{d} values of GLPG0634 on JAK1, JAK2, and JAK3 were determined by measuring the competition of GLPG0634 with a fluorescently labeled ATP mimetic. IC_{50} values corresponding to 50% probe displacement were obtained and K_{d} values calculated according to the Cheng-Prusoff equation.

**Table II. Potency and selectivity of GLPG0634 in cellular assays**

<table>
<thead>
<tr>
<th>JAKs Involved</th>
<th>Cell Type</th>
<th>Trigger</th>
<th>Readout</th>
<th>IC_{50} (nM)</th>
<th>pIC_{50} ± SEM</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAK1–JAK3</td>
<td>THP-1</td>
<td>IL-4</td>
<td>pSTAT6</td>
<td>154, 203</td>
<td>6.75 ± 0.06</td>
<td>2</td>
</tr>
<tr>
<td>JAK1–JAK3</td>
<td>NK-92</td>
<td>IL-2</td>
<td>pSTAT5</td>
<td>148, 757, 367</td>
<td>6.46 ± 0.12</td>
<td>3</td>
</tr>
<tr>
<td>JAK1–TYK2</td>
<td>U2OS</td>
<td>IFN-αb</td>
<td>pSTAT1</td>
<td>494, 436</td>
<td>6.33 ± 0.03</td>
<td>2</td>
</tr>
<tr>
<td>JAK1–JAK2</td>
<td>HeLa</td>
<td>OSM</td>
<td>STAT1 reporter</td>
<td>1,045</td>
<td>6.01 ± 0.07</td>
<td>4</td>
</tr>
<tr>
<td>JAK1–JAK2</td>
<td>U2OS</td>
<td>IFN-γ</td>
<td>pSTAT1</td>
<td>3,364</td>
<td>5.47</td>
<td>1</td>
</tr>
<tr>
<td>JAK2</td>
<td>TF-1</td>
<td>IL-3</td>
<td>pSTAT5</td>
<td>3,524</td>
<td>5.45</td>
<td>1</td>
</tr>
<tr>
<td>JAK2</td>
<td>BaF3</td>
<td>IL-3</td>
<td>Proliferation</td>
<td>4,546</td>
<td>5.34 ± 0.04</td>
<td>3</td>
</tr>
<tr>
<td>JAK2</td>
<td>U7-EPO</td>
<td>EPO</td>
<td>pSTAT5</td>
<td>&gt;10,000</td>
<td>&gt;5</td>
<td>2</td>
</tr>
<tr>
<td>JAK2</td>
<td>22rV1</td>
<td>PRL</td>
<td>pSTAT5</td>
<td>&gt;10,000</td>
<td>&gt;5</td>
<td>2</td>
</tr>
</tbody>
</table>

IC_{50} values in cellular assays were determined by plotting the compound concentration versus the effect on the readout. The pIC_{50} is defined as the negative of the log_{10} of the compound concentration having a half maximal effect on the readout.
dosed once daily by oral gavage, initially at doses of 3, 10, and 30 mg/kg. Because high efficacy was obtained at 3 mg/kg, two follow-up studies were carried out with lower doses. Even at 0.1 mg/kg, a statistically significant effect was observed in the clinical score from the fifth day of dosing onward (Fig. 4B). Data obtained from each of the three studies were normalized to the corresponding vehicle data, and a meta-analysis of the three studies was performed (Fig. 4A, 4C, 4D). A meta-analysis of the steady-state pharmacokinetics from the rat CIA studies showed dose-proportional increases of maximum blood concentration (C_{max}) and area under curve between 0.3 and 30 mg/kg and correlated with the dose-dependent efficacy (Fig. 4A). A rapid absorption was observed for GLPG0634, with maximal plasma levels achieved ∼1 h after dosing, for all dose levels tested. Half-life is ∼4–5 h and is independent of dose level. A dose-dependent effect was observed in all pharmacodynamic readouts. The doses of 1, 3, and 10 mg/kg GLPG0634 reduced the clinical score to the same extent as etanercept at endpoint. Different from the GLPG0634 doses tested, the high dose of etanercept normalized the clinical score already from the start of dosing (Fig. 4B). Statistically significant reduction of the clinical score at endpoint was obtained for all doses (Fig. 4C). Protection from bone damage was evidenced by a dose-dependent reduction of the Larsen score obtained after x-ray analysis of the hind paws, with significant effect from 3 mg/kg and onward (Fig. 4D). Similar efficacy was obtained for GLPG0634 as for etanercept. Histological analysis of the rat paws was performed in a specific region of interest including the talus, navicular, and cuneiform bones (Fig. 4E–H). As compared with the vehicle control group (Fig. 4E), etanercept and GLPG0634 groups (Fig. 4E, 4G, 4H) showed a marked reduction of the infiltration of inflammatory cells while protecting the articular cartilage and bone from 1 mg/kg onward.

Confirmation of the therapeutic potential of GLPG0634 in CIA in the rat was provided by studying the compound in the similar model in the mouse. In addition to the parameters measured in the rat, mouse blood and paw samples were taken with the purpose of obtaining detailed insight in the mechanism of action of GLPG0634 in vivo. Dose selection was performed in a dose range–finding experiment (data not shown), and a dose of 50 mg/kg twice daily orally was selected for the mechanistic studies. Fig. 5A shows that the 50 mg/kg dose provided full protection against inflammation as judged by analysis of the clinical score of paws. Histological analysis of the mice paws showed that GLPG0634 protected bone and cartilage from degradation (Fig. 5B). Immunohistochemistry performed on the same samples showed that GLPG0634 effectively reduced infiltration of T cells (CD3^+ cells) and macrophages (F4/80) by guest on April 17, 2017 http://www.jimmunol.org/ Downloaded from

### Table III. Potency and selectivity determination of GLPG0634 in human WBAs

<table>
<thead>
<tr>
<th>Assay</th>
<th>IL-6/pSTAT1</th>
<th>IL-2/pSTAT5</th>
<th>IFN-α/pSTAT1</th>
<th>GM-CSF/pSTAT5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>JAK involved</strong></td>
<td><strong>Cell type</strong></td>
<td>CD4^+</td>
<td>CD4^+</td>
<td>CD4^+</td>
</tr>
<tr>
<td>GLPG0634</td>
<td>6.201 ± 0.092 (629; 7)</td>
<td>5.747 ± 0.043 (1,789; 5)</td>
<td>5.948 ± 0.021 (1,127; 6)</td>
<td>4.758 ± 0.214 (17.453; 7)</td>
</tr>
<tr>
<td>Tofacitinib</td>
<td>7.130 ± 0.119 (74; 16)</td>
<td>7.473 ± 0.033 (33; 9)</td>
<td>7.120 ± 0.040 (76; 6)</td>
<td>6.130 ± 0.179 (740; 7)</td>
</tr>
<tr>
<td>INCB028050</td>
<td>7.631 ± 0.169 (23.4; 4)</td>
<td>ND</td>
<td>ND</td>
<td>7.195 ± 0.055 (63.8; 6)</td>
</tr>
</tbody>
</table>

IC_{50} values for inhibition of cytokine-induced STAT phosphorylation were determined by measuring STAT phosphorylation in CD4^+ cells or CD33^+ cells using flow cytometry in whole blood. The pIC_{50} (defined as the negative of the log_{10} of the compound concentration having a half maximal effect on the readout) was measured for each volunteer and averaged. Data are represented as mean pIC_{50} ± SEM and IC_{50,a} are derived from the mean pIC_{50} values. JAK1 versus JAK2 selectivity (outer column) was determined by comparing potencies measured in the IL-6/pSTAT1 and GM-CSF/pSTAT5 assays.

ND, Not determined.
80^4 cells) in the paw (Fig. 5C). The effects on clinical score, bone and cartilage protection, and cell infiltration were similar to the results obtained by etanercept. Gene expression studies were carried out in WBCs and paws. Expression levels increased in arthritic versus healthy animals for all genes tested (Fig. 5D, 5F, 5G). In paws, GLPG0634 reduced the levels of inflammatory and metalloprotease genes previously linked to disease progression, explaining the beneficial role of GLPG0634 in the mouse CIA model (Fig. 5D). The mRNA levels of RANKL were also reduced in line with the decrease in the bone lesion score, suggesting that GLPG0634 might protect against bone degradation by reducing the formation and activity of osteoclasts. Cytokine levels in sera from the CIA mice were measured using Luminex technology. As for the gene expression studies, the levels of the protein markers under study were raised in the serum of diseased versus healthy animals, with the exception of stem cell factor (Fig. 5E, Supplementary Table I). GLPG0634 decreased the serum levels of all cytokines and chemokines measured, including IL-6, IP-10, XCL1, and MCP-1 (Fig. 5E). These observations indicate that GLPG0634 might affect inflammatory cytokine signaling and chemotraction of T cells and monocyte/macrophages by reducing these cytokine and chemokine levels. At the mechanistic level, the reduction of Mx1 and Mx2 mRNA levels by GLPG0634 was also observed in mouse paws (Fig. 5F), as observed in the rat (Fig. 3). Of interest, the changes in Mx1 and Mx2 gene expression in WBCs were not altered by etanercept treatment (Fig. 5G), showing that GLPG0634 specifically impacts JAK1 signaling.

**Discussion**

In recent years significant advances have been made in understanding the link between the different JAK family members and their involvement in autoimmune, inflammatory, and oncological diseases. The first generation of small-molecule inhibitors has further substantiated the therapeutic potential of JAK inhibitors in the aforementioned diseases. In this study we examined GLPG0634, a novel and selective JAK inhibitor that demonstrates selectivity for JAK1 in a cellular environment. GLPG0634 was identified in a kinase-focused library screen and belongs to the triazolopyridine compound class. Characterization of GLPG0634 at the biochemical level indicated a selective inhibition of JAK1 and JAK2 over JAK3 and TYK2, whereas cellular and WBAVs revealed a selectivity for JAK1- over JAK2-dependent signaling in a cellular environment. GLPG0634 efficiently blocks cytokine-induced signaling cascades involving JAK1 in several cell lines as well as in human primary cells. Moreover, Th1, Th2, and Th17 differentiation driven by cytokine cocktails, including JAK1-dependent cytokines such as IL-2, IL-4, and IL-6, is also inhibited by GLPG0634 (17, 18). These in vitro findings translate to pharmacodynamic readouts in rodents showing that JAK1 signaling is blocked in vivo as measured by a reduction of Mx2 mRNA levels. Furthermore, GLPG0634 dose-dependently reduces inflammation, cartilage, and bone degradation in the CIA model in rats and mice.

The biochemical selectivity profile of GLPG0634 (rank order of potency JAK1 ~ JAK2 > TYK2 > JAK3) was a poor predictor of the selectivity determined in cellular and WBAVs. TYK2 enzyme activity in biochemical assays was inhibited with 11- and 4-fold lower potency versus JAK1 and JAK2, whereas JAK3 enzyme activity was inhibited with a much lower potency. This difference did not translate into a higher cellular potency of inhibiting INF-α/JAK1/TYK2 signaling versus IL-2/JAK1/JAK3 signaling (Tables II, III). It indicates that JAK1 inhibition is in large part responsible for the potency of GLPG0634 in a cellular environment. A JAK inhibitor likely requires equipotent inhibition of JAK1 and TYK2 or JAK3, or even inverse selectivity for the latter enzymes to surpass potency derived from JAK1 inhibition. This is demonstrated for tofacitinib, which shows 3-fold selectivity for JAK3 over JAK1 in biochemical assays and inhibits IL-2–induced STAT5 phosphorylation twice more potently than IL-6–induced STAT1 phosphorylation (Table III) (9). The observation that GLPG0634 revealed a high selectivity for JAK1 over JAK2 in cellular and human WBAs was unexpected. Interestingly, a similar selectivity shift was observed for tofacitinib but not for baricitinib when testing these molecules in parallel with GLPG0634 (Table III). This is remarkable, as tofacitinib and baricitinib share the same chemical scaffold and show similar potencies toward JAK1 and JAK2 in biochemical assays (9, 10, 28). At present no univocal explanation for the discrepancy between the biochemical and cellular/whole blood JAK inhibition profiles can be provided. A potential explanation may be linked to the differences between the biochemical and cellular assay formats. First, the biochemical assay relies on kinase activity of a purified truncated protein containing the C-terminal quarter of the JAK proteins, including the JH1...
kinase domain. In contrast, in a cellular environment wild-type full-length JAKs comprise the regulatory JH2 pseudokinase domain, Src homology 2 domains (JH3–JH4), and the amino terminal (NH2) FERM domain (JH4–JH7). Additionally, JAKs are part of a larger complex, including the cytoplasmic receptor tails, STATs, and other proteins (2, 4). Second, the endogenous JAKs are subject to posttranslational modifications such as phosphorylation. A differential tyrosine phosphorylation status can give rise to different IC50 values as exemplified for the non- versus monophosphorylated forms of the TYK2 kinase domain (29).

Third, differential negative or positive feedback mechanisms for JAK1- versus JAK2-dependent signaling by means of phosphatases, members of the SOCS or SH2B families, can have a different impact on the amplitude and kinetics of JAK enzyme activity and signaling output (30–33). Finally, different small molecules might induce subtle changes in the three-dimensional space of the ATP-binding pockets of the JAKs, leading to differential kinase activity and/or protein–protein interactions. Crystallography of JAK proteins harboring more than the JH1 kinase domain in complex with small molecule inhibitors will provide more insight here.

Oral administration of GLPG0634 in the mouse and rat resulted in good plasma exposure. GLPG0634 plasma levels in the rat could be correlated with reduction of Mx2 mRNA levels in WBCs reflecting target engagement and inhibition of JAK/STAT signaling in vivo. Additional testing of GLPG0634 in vivo in rodent CIA models in a therapeutic setting revealed that daily oral administration resulted in a dose-dependent reduction of inflammation and protected bone and cartilage from degradation. In view of the pathological roles of Th1 effector T cells in chronic inflammation and autoimmune disorders and the dose-dependent reduction of

<table>
<thead>
<tr>
<th>Parameter (Unit)</th>
<th>Mouse 1 mg/kg i.v.</th>
<th>Mouse 5 mg/kg Orally</th>
<th>Rat 1 mg/kg i.v.</th>
<th>Rat 5 mg/kg Orally</th>
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<tr>
<td>Cmax or C0 (ng/ml)</td>
<td>637</td>
<td>920</td>
<td>1407 (28)</td>
<td>310 (33)</td>
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<td>Tmax (h)</td>
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<td>1893</td>
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<tr>
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<td>Vss (l/kg)</td>
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<td>1.8 (3)</td>
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<td>F (%)</td>
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Table IV. Rodent pharmacokinetics for GLPG0634

**FIGURE 3.** Pharmacokinetics/pharmacodynamics modeling of GLPG0634 in healthy male Sprague Dawley rats. Mx2 mRNA levels in WBCs and GLPG0634 plasma levels were determined at various time points after administration of four daily oral doses of 1 mg/kg (A, B) or 10 mg/kg (C, D) GLPG0634 in one experiment. Three animals were used per time point. Mx2 mRNA fold inhibition levels were calculated versus vehicle-treated samples after normalization to β-actin mRNA (left y-axis). GLPG0634 plasma levels are depicted on the right y-axis. (A and C) Fold inhibition of basal Mx2 mRNA levels. (B and D) Fold inhibition of ex vivo IFN-α–induced Mx2 mRNA levels. *0.05 > p > 0.01 versus vehicle, Student t test.
Th1 differentiation by GLPG0634 in vitro, its efficacy in the CIA models is in line with these in vitro observations (34). Quantitative PCR analysis of disease-related inflammatory markers in blood and paws showed a pronounced reduction of these markers by GLPG0634. This was confirmed at the protein level by measuring cytokine levels in sera of arthritic mice treated with GLPG0634 versus vehicle-treated animals. Histological and x-ray (Larsen score) analysis of mice and rat paws revealed reduced bone and cartilage degradation coinciding with reduced infiltration by T cells and macrophages as shown in the mouse CIA study. The efficacy of GLPG0634 in the murine CIA models and the changes in the disease-related biomarkers are of similar magnitude as the relatively high dose of etanercept. Differing from the efficacy observed in rodent CIA models, only a moderate efficacy was obtained in the mouse experimental allergic encephalomyelitis model of multiple sclerosis (Supplemental Fig. 1). Because high efficacy in this model likely requires passage through the blood–brain barrier, the observation that GLPG0634 is a substrate for P-glycoprotein (efflux ratio in Caco2 permeability assay decreased from 16 to 6 in the presence of verapamil; data not shown) could explain its moderate activity.

Observing efficacy for GLPG0634 in the rat CIA model at doses as low as 0.1 and 0.3 mg/kg was surprising. This is unlikely due to off-target effects in view of the clean profile of GLPG0634 in kinase panels. GLPG0634 showed >100-fold selectivity over other kinases representing the human kinome (175 kinases tested) with the exception of FLT3, FLT4, and CSF1R, for which selectivity still was >25-fold (Supplemental Table II). Speculative explanations include...
tissue-specific compound accumulation, active metabolites, or even other mechanisms, but a final explanation remains to be determined. When GLPG0634 was dosed at 1 mg/kg or higher pharmacokinetic and pharmacodynamic data correlated well. The $C_{\text{max}}$ levels measured for the 1 and 3 mg/kg doses of GLPG0634 in the rat pharmacokinetics/pharmacodynamics and CIA in vivo studies (240–844 nM; Figs. 3A, 3B, 4A) were close to or exceeded the JAK1-dependent IL-6/pSTAT1 human WBA IC$_{50}$ value (623 nM; Table III). Preliminary evidence obtained by measuring a JAK1 biomarker in a phase I clinical trial for GLPG0634 indicated that target engagement in humans can be measured for doses corresponding to the 1 mg/kg dose from the CIA and Mx2 in vivo studies (35). A similar conclusion was reached for baricitinib when comparing WBA IC$_{50}$ values with efficacy in a rat arthritis model (10). Also, the clinically relevant dose of 5 mg twice daily for tofacitinib resulted in $C_{\text{max}}$ levels in human volunteers (41–52 ng/ml) that are ~2-fold above the JAK1-dependent WBA IC$_{50}$ value (23 ng/ml or 74 nM; Table III) and will not completely inhibit JAK1 and/or JAK3 for most of the day (36). Hence, incomplete inhibition of JAK1 and the wide range of cytokine signaling it serves can provide therapeutic efficacy, thereby decreasing the risk of potential JAK1-mediated side-effects such as immune suppression.

The high selectivity of GLPG0634 for JAK1 versus JAK2 observed in vitro is supported by a number of observations made in patient studies. After dosing GLPG0634 for 10 d to healthy volunteers up to 450 mg once daily, no relevant findings on hematology (including reticulocytes), biochemistry (including cholesterol and lipids), or other safety parameters (electrocardiogram, vital signs) were noted (37). At this dose, JAK1 signaling was suppressed for 24 h whereas JAK2 signaling was not influenced (37). Moreover, dosing GLPG0634 to 24 RA patients for 4 wk at daily doses of 200 mg showed efficacy on ACR20 scores and on the secondary endpoints of DAS28 and serum C-reactive protein levels without adverse events (38). Instead of anemia, which could have been indicative for inhibition JAK2 signaling impairing hematopoiesis, a small increase in hemoglobin levels was noted as expected with improvement in disease.

In conclusion, GLPG0634 is a promising drug candidate for the future treatment of autoimmune and inflammatory disorders such as RA.

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Disclosures
All authors are employees of the Galapagos group (which includes BioFocus) and are eligible to receive stock options.

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