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

Luc Van Rompaey,* René Galien,† Ellen M. van der Aar,* Philippe Clement-Lacroix,† Luc Nelles,* Bart Smets,* Lièn Lepescheux,‡ Thierry Christophe,* Katja Conrath,* Nick Vandegehinste,* Béatrice Vayssiere,‡ Steve De Vos,* Stephen Fletcher,*¹ Reginald Brys,* Germain Crevecoeur One,* Jean H. M. Feyen,* and Christel Menet*

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: 600–600.

The online version of this article contains supplemental material.

Abbreviations used in this article: CII, collagen type II; CIA, collagen-induced arthritis; Cmax, maximum blood concentration; EPO, erythropoietin; OSM, oncostatin M; PRL, prolactin; RA, rheumatoid arthritis; RT, room temperature; siRNA, small interfering RNA; WBA, whole blood assay.

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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× the experimental K₅₀ value and the substrate concentration (UL-conjugated JAK-1(Tyr1022) peptide) corresponded to the experimentally determined K₅₀ 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 Lacquer detection buffer (PerkinElmer). Compound IC₅₀ values were determined by preincubating the enzyme with compound at RT for 60 min, prior to the addition of ATP.

K₅₀ determination. Dissociation constants were determined at Proteros Biotostructures ( Martinsried, Germany). Proprietary fluorescently labeled ATP mimetics with fast dissociation rates (PRO13, PRO14, and PRO13 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 hydroxyectoine (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 K₅₀ values were calculated according to the Cheng–Prusoff equation.

Cellular assays

STAT5 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), 50 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 50 mM NaF, and 30 mM sodium pyrophosphate, 10% glycerol buffer containing phosphatase/protease inhibitor cocktails, and centrifuged. Cell lysate (180 μg) was preincubated with compound at RT for 1 h, stimulated with EPO (1 U/ml) for 20 min, and processed for AlphaScreen analysis. UT-7 erythropoietin (EPO) cells (EPO-dependent derivative of UT-7; Centocor) were preincubated with compound at RT for 1 h, stimulated with EPO (1 U/ml) for 20 min, and processed for AlphaScreen analysis. UT-7 erythropoietin (EPO) cells (EPO-dependent derivative of UT-7; Centocor) were preincubated with compound at RT for 1 h, stimulated with EPO (1 U/ml) for 20 min, and processed for AlphaScreen analysis. UT-7 erythropoietin (EPO) cells (EPO-dependent derivative of UT-7; Centocor) were preincubated with compound at RT for 1 h, stimulated with EPO (1 U/ml) for 20 min, and processed for AlphaScreen analysis. pSTAT5 was measured using proprietary fluorescently labeled ATP mimetics with fast dissociation rates (PRO13, PRO14, and PRO13 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 hydroxyectoine (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 K₅₀ values were calculated according to the Cheng–Prusoff equation.

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 (4 ng/ml; R&D Systems), universal IFN-α (1000 U/ml; PBL Biomedical Laboratories), recombinant human GM-CSF (20 pg/ml; PeproTech), or vehicle (PBS plus 0.1% [v/v] BSA) at 37°C for 20 min and treated with prewarmed 1× lysis buffer (BD Biosciences) to lyse RBCs and fix leukocytes. Cells were permeabilized with 100% methanol and incubated with anti-pSTAT1 and anti-CD4 (IL-6– and IFN-γ–triggered samples), anti-pSTAT5 and anti-CD4 Abs (IL-6–triggered samples), or anti-pSTAT5 and anti-CD3 Abs (GM-CSF–triggered samples) (all Abs were from BD Biosciences) at 4°C for 30 min, washed once with PBS 1×, and analyzed on a FACScanto II flow cytometer.

Pharmacokinetics

Formulations. GLPG0634 was formulated in polyethylene glycol 2000/90% 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 to the jugular vein for administration 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 were approved by the local ethical committee (Galapagos).

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 GAPDH L-MAX control siRNAs (Dharmacon) using Lipofectamine RNAMAX transfection reagent from Invitrogen.

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
isoflurane anesthesia. Lithium heparin was used as anticoagulant and blood was taken at 0.5, 0.25, 0.5, 1, 3, 5, and 8 h (i.v. route) and 0.25, 0.5, 1, 3, 5, 8, and 24 h (by mouth).

GLPG0634 plasma concentrations were determined by liquid chromatography–tandem mass spectrometry with a lower limit of quantification of 2 ng/ml. Pharmacokinetic parameters were calculated by noncompartmental analysis using WinNonlin software (Pharsight, version 5.2).

In vivo pharmacology

Rodent CIA models. Animals. Dark Agouti rats (females, 7–8 wk old) and DBA/1J mice (male, 6 wk old) were obtained from Janvier (Lavil, France). Materials. CFA and IFA were purchased from Difco (Detroit, MI). Bovine collagen type II (CII) was obtained from Chondrex (Redmond, WA). 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 intradermally 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 0.01–30 mg/kg. The TNF-α blocker etanercept (Wyeth Pharmaceuticals, Taplow, U.K.) was administered three times per week at 10 mg/kg i.p. 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 etanercept dosed three times a week i.p. as measured by clinical score, inflammation, bone resorption, pannus, and cartilage damage. At day 7 or 11, 200 μl blood was collected from the retro-orbital puncture with lithium heparin as anticoagulant at predose and 1, 3, and 6 h (n = 2 or 3 time point) for steady-state pharmacokinetics analysis. At sacrifice, hind paws were removed for x-ray analysis and histological examination. A Tukey multiple comparison test was used to perform a meta-analysis of three studies carried out for GLPG0634. The score of each rat was divided by the average score obtained for vehicle in the same readout and study and multiplied by 100. Relative scores were averaged per rat, and animals presenting 0% of studies that received the same dose. For mouse CIA experiments, the IFA/CII emulsion (0.2 ml) was injected intradermally at the base of the tail at day 1 and again at day 21. This immunization method was modified from published methods (20). 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, 2.4 ± 0.6; 10 mice/treatment group) over the dose range 50 mg/kg twice daily. Administration of etanercept and pharmacodynamic and pharmacokinetic analyses were essentially carried out as described for the rat CIA model.

Clinical assessment of arthritis. The individual clinical score was obtained by summing the scores recorded for each limb. Arthritis was scored from grade 0 to 4 according to an established method (19).

Larsen score. X-ray imaging was performed for the hind paws of each individual animal. An identity number was randomly assigned to each of the images, and the severity of bone erosion was ranked by two independent blinded scorers as described earlier (21).

Histology. For rat CIA studies, the hind paws were collected, fixed in 10% formaldehyde solution (Eurobio, Paris, France) for 24 h, and decalcified in RDO solution (Eurobio) at 4°C for 3 d. Three series of 5-μm sections were made at 100-μm intervals from the paw middle part and stained with Goldner’s trichrome. The inflammation status and skeletal tissue damage were examined by light microscope (Provis, Olympus). For mouse CIA studies, the hind paws were collected, fixed in 3.7% (v/v) formaldehyde solution for 24 h, and decalcified in Osteosoft solution (VWR International, Val de Fontenay, France) for 24 d. Each paw was cut in two parts according to the sagittal axis and embedded in paraffin. Six series of 4-μm thick sections were collected. One series of sections was stained with safranin O-light green for morphological examination and disease scoring. Disease score was obtained by double-blinded histopathologist as described by Ratcliffe et al. (21). Statistical analysis was performed using an ANOVA unpaired test with *p < 0.05, **p < 0.01, and ***p < 0.001 versus vehicle group. Immunohistochemistry was performed with Abs detecting macrophage (anti-F4/80, sc-59171; Santa Cruz Biotechnology) and T cell (anti-CD3; Dako) markers. Biotinylated horse anti-rabbit Ab and avidin-linked peroxidase (Vectastain Universal Elite ABC kit;Vector Laboratories) were used to detect the binding of antibody stained in brown color. Immunopositive cells were quantified by image analysis and related to the total number of cells. Statistical analysis was performed by using an ANOVA unpaired test with *p < 0.05, **p < 0.01, and ***p < 0.001 versus vehicle group.

Gene expression and pharmacokinetic/pharmacodynamic modeling in rodent blood. Fed male Sprague Dawley rats (180–200 g) received four daily oral doses of vehicle or GLPG0634 at 1 or 10 mg/kg. Blood samples were taken on the fourth day of dosing at 0.5, 1, 2, 6, and 24 h after dose via decapitation (n = 3/time point). GLPG0634 plasma concentrations were determined by liquid chromatography–tandem mass spectrometry. Each blood sample (2 ml) was divided into 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 RLT (Qiagen). Total RNA was extracted using the QIAamp RNA Blood Mini kit (Qiagen) and 500 ng was reverse transcribed using TaqMan RT (Applied Biosystems) with oligo(dT) priming. Five microliters of 5% diluted cDNA preparations was 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 (Life Technologies) with random hexamers. Quantitative PCR reactions were performed using QuantFast SYBR Green PCR Master mix (Qiagen) and gene-specific primer pairs for β-actin (Glyceraldehyde) 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 were expressed as ΔΔCT 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). Total RNA was isolated by phenol/chloroform extraction and precipitated with 1 vol. ethanol, rinsed with 70% ethanol, and air dried. RNA quality and concentration were assessed on a Bioanalyzer 2100 (Agilent Technologies) with an RNA 6000 nanochip. cDNA was synthesized from 1 μg of total RNA using 5 μl of 5X First-Strand Buffer, 0.5 μl of dNTPs, 2 μl of 1 M DTT, 2 μl of tRNA, 1 μl of random primers, 0.8 μl of AmpliScribe T7 transcriptase (Epicentre), and 0.2 μl of T7 RNAP (Epicentre) and incubated at 37°C for 15 min and then at 65°C for 5 min. The cDNA was diluted to 10 μl and stored at −20°C.

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 potency of JAK1 > JAK2 > TYK2 > JAK3 (Table 1). IC50 values determined in tyrosine kinase inhibition assays correlated with KD values determined in ligand displacement assays (Table 1).

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/γc signaling and IFN-α2B–induced JAK1/ TYK2 type II receptor signaling most potently. IC50 values ranged from 150 to 760 nM (Table 1). 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
Inhibitors, tofacitinib and baricitinib, were also tested in both assays over JAK2-dependent signaling (Table III). Two clinical JAKs corresponding to the ∼30-fold selectivity for inhibition of JAK1- over JAK2-dependent signaling (Table III). Two clinical JAK inhibitors, tofacitinib and baricitinib, were also tested in both assays and showed high potency. A respective 10- and 3-fold JAK1 over JAK2 selectivity was determined even though both compounds equipotently inhibited JAK1 and JAK2 in biochemical assays (9, 10). These data indicate that GLPG0634 selectively inhibits JAK1-dependent signaling in a cellular environment and is more JAK1 selective than are tofacitinib and baricitinib. GLPG0634 also inhibited the STAT5 phosphorylation activated by IL-2 and STAT1 phosphorylation by IFN-α, although with a lower potency than STAT1 phosphorylation by IL-6. Tofacitinib inhibited IFN-α signaling with similar potency as IL-6/STAT1 signaling but inhibited IL-2/STAT5 signaling with higher potency (Table III). These observations confirm the JAK1/JAK3 inhibition profile of tofacitinib in cells reported by Meyer et al. (9). The WBA data confirm the observations from the cellular assays showing that GLPG0634 preferentially inhibits JAK signaling complexes containing JAK1.

To further explore the consequences of cytokine inhibition, the effect of GLPG0634 toward Th1 and Th2 differentiation was analyzed by measuring IFN-γ or IL-13 mRNA expression levels (Fig. 2). As expected, GLPG0634 dose-dependently inhibited the differentiation of Th2 cells mediated by IL-4, a cytokine that signals through JAK1 and JAK3. GLPG0634 also inhibited Th1 differentiation with similar potencies of 1 μM or lower. Although Th1 commitment is initiated by IL-12, a cytokine signaling through TYK2 and JAK2, the primary effect of GLPG0634 on Th1 differentiation is likely through inhibition of JAK1/JAK2-mediated signaling of IFN-γ. Inhibition of IFN-γ signaling alone was previously found to be sufficient to reduce T-bet, the master transcriptional regulator critical for Th1 differentiation, and IFN-γ in Th1 cells and it was proposed as a mechanism explaining the inhibitory effect of tofacitinib on Th1 cell differentiation (3). GLPG0634 was also tested for the ability to inhibit Th17 differentiation driven by a mixture of TGF-β, IL-23, and proinflammatory cytokines (IL-6 and IL-1β), all shown to be essential for human Th17 differentiation (18). GLPG0634 inhibited Th17 differentiation under these conditions, although with lower potency than Th1 and Th2 differentiation (Fig. 2).

The pharmacokinetics of GLPG0634 was determined in rats and mice. Following i.v. administration, GLPG0634 displayed a low to moderate plasma clearance, depending on the species tested (Table IV). In mice, the total clearance represented 58% of the liver blood flow, and in rats it represented 41%. Steady-state volume of distribution ranged from ∼1.7 l/kg in rats to 6 l/kg in mice, implying a significant species difference in volume of distribution. Half-life observed after oral administration was 1.7 h in mice and 3.9 h in rats. Following oral administration, the absolute bioavailability was moderate in rats (∼45%) and high in mice (∼100%). Because GLPG0634 was well exposed in several species, inhibition of JAK signaling in vivo was evaluated in a biomarker assay. In WBCs, Mx2 mRNA levels are modulated by IFN-α in Th1 cells and IFN-γ or IL-13 mRNA expression levels (Fig. 3). Doses were dosed orally with GLPG0634 and both basal and ex vivo IFN-α–induced Mx2 mRNA levels were measured. A statistically significant reduction of normalized Mx2 mRNA levels were observed in WBCs of rats and mice treated with GLPG0634 compared to vehicle-treated controls. The WBA data confirm the pharmacokinetics results, indicating that GLPG0634 has a long-lasting inhibitory effect on JAK signaling in vivo, which is consistent with the in vitro studies and the clinical observations of Meyer et al. (9).

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

<table>
<thead>
<tr>
<th>Recombinant Human Kinase</th>
<th>IC50 (nM, ± SEM; n = 2–4)</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAK1</td>
<td>10 ± 0.8</td>
<td>11</td>
</tr>
<tr>
<td>JAK2</td>
<td>28 ± 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>

IC50 values for inhibition of recombinant JAK1, JAK2, JAK3, and TYK2 by GLPG0634 were determined by measuring the incorporation of phosphate into an ULight-JAK1(Tyr1023) peptide using an europium-labeled anti-phosphotyrosine Ab. The Kd values of GLPG0634 on JAK1, JAK2, and JAK3 were determined by measuring the competition of GLPG0634 with a fluorescently labeled ATP mimetic. IC50 values corresponding to 50% probe displacement were obtained and Kd values calculated according to the Cheng-Prusoff equation.

ND, Not determined

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>IC50 (nM)</th>
<th>pIC50 ± 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>6.35 ± 0.03</td>
<td>2</td>
</tr>
<tr>
<td>JAK1–TYK2</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>2,364</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>U77-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>

IC50 values in cellular assays were determined by plotting the compound concentration versus the effect on the readouts mentioned. The pIC50 is defined as the negative of the log10 of the compound concentration having a half maximal effect on the readout.
levels was measured upon dosing 1 and 10 mg/kg with more pronounced reductions at the dose of 10 mg/kg (Fig. 3). For the basal Mx2 mRNA levels, a hysteresis between compound peak plasma levels and maximal inhibition of basal Mx2 mRNA levels was observed as expected.

The therapeutic potential of GLPG0634 for RA was evaluated in a well-accepted animal model for RA (19, 25–27). GLPG0634 was 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 obtained 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 mouse 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; 4G, 4H) showed a marked reduction of the infiltration of inflammatory cells while protecting the articular cartilage and bone from 1 mg/kg onward.

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>JAK involved</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell type</td>
<td>JAK1 CD4+</td>
<td>JAK1 &gt; JAK3</td>
<td>JAK1–TYK2 CD4+</td>
<td>JAK2 CD33+</td>
</tr>
<tr>
<td>Compounds</td>
<td>pIC50 ± SEM (IC50 [nM]; n)</td>
<td>pIC50 ± SEM (IC50 [nM]; n)</td>
<td>pIC50 ± SEM (IC50 [nM]; n)</td>
<td>pIC50 ± SEM (IC50 [nM]; n)</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>INCBO28050</td>
<td>7.631 ± 0.169 (23; 4)</td>
<td>ND</td>
<td>ND</td>
<td>7.195 ± 0.055 (63; 8)</td>
</tr>
</tbody>
</table>

pIC50 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 pIC50 (defined as the negative of the log10 of the compound concentration having a half maximal effect on the readout) was measured for each volunteer and averaged. Data are represented as mean pIC50 ± SEM and IC50s are derived from the mean pIC50 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⁺ 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 were increased in arthritic versus healthy animals for all genes tested (Fig. 5D, 5F, 5G). In paws, GLPG0634 reduced the levels of inflammatory and metallopeptase 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, Supplemental 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 WBAs 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 WBAs. 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 mono- or diphosphorylated 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 IV. Rodent pharmacokinetics for GLPG0634

<table>
<thead>
<tr>
<th>Parameter (Unit)</th>
<th>Mouse 1 mg/kg i.v.</th>
<th>5 mg/kg Orally</th>
<th>Rat 1 mg/kg i.v.</th>
<th>5 mg/kg Orally</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax or C₀ (ng/ml)</td>
<td>637</td>
<td>920</td>
<td>1407 (28)</td>
<td>310 (33)</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>0.5</td>
<td>1.7</td>
<td>1.6 (3)</td>
<td>3.9</td>
</tr>
<tr>
<td>Area under curve: 0–24 h (ng/h/ml)</td>
<td>347</td>
<td>1893</td>
<td>739 (2)</td>
<td>1,681 (8)</td>
</tr>
<tr>
<td>T₁/₂ (h)</td>
<td>2.5</td>
<td>1.7</td>
<td>1.6 (3)</td>
<td>3.9</td>
</tr>
<tr>
<td>Cl (l/h/kg)</td>
<td>2.9</td>
<td>1.4 (2)</td>
<td>1.8 (3)</td>
<td>45</td>
</tr>
<tr>
<td>Vss (l/kg)</td>
<td>6</td>
<td>∼100</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>F (%)</td>
<td>100</td>
<td>45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pharmacokinetics of GLPG0634 in mouse and rat (mean values of n = 3 [CV]) after a single oral (5 mg/kg) or i.v. (1 mg/kg) administration.

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

**FIGURE 4.** GLPG0634 dose-dependently prevents disease progression in the therapeutic rat CIA model. (A) Meta-analysis of plasma exposure of GLPG0634 at steady-state. Total concentrations are provided on the y-axis. (B) Treatment was started 18 d after the first collagen injection and after randomization of animals in treatment groups (day 0) for 15 d. The clinical score readout is shown for the CIA study that included the lowest doses of GLPG0634. (C and D) Meta-analysis of combined data obtained for the three studies after 15 d of treatment. E, etanercept; V, vehicle. GLPG0634 doses are indicated on the x-axis. The numbers of animals used to obtain a meta-analysis score for V, E, 0.1, 0.3, 1, 3, 10, and 30 mg/kg were 29, 10, 20, 20, 29, 20, 10, and 29, respectively. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle, Student t test. (C) Difference between the clinical score at day 0 and day 15. (D) Larsen score: bone degradation determined by x-ray analysis at end point. (E–H) Histological analysis of rat hind paws was performed for vehicle (E), etanercept (F), GLPG0634 (1 mg/kg) (G), and GLPG0634 (10 mg/kg) (H) treatment groups. Sagittal sections comprising the talus (T), navicular (N), and the cuneiform (C) bones were stained with Goldner trichrome. Original magnification ×20. Despite a remaining inflammation (black asterisk) seen for all animals, the articular cartilage and bone were well preserved (black arrows) in the two groups dosed with GLPG0634 when compared with CIA-vehicle group (red arrow).
and in circulating leukocytes at the end of the 2-wk treatment (induced genes Mx1 and Mx2 in paws after a single compound administration.)

4 h after Luminex measurement of serum concentration of several markers of inflammation (IL-6, IP-10/CXCL10, XCL1/lymphotactin, MCP-1/CCL2) were 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_{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.

References

JAK1 INHIBITOR GLPG0634 IS EFFICACIOUS IN RODENT CIA MODELS


