Tyrosine Kinase 2-mediated Signal Transduction in T Lymphocytes Is Blocked by Pharmacological Stabilization of Its Pseudokinase Domain

John S. Tokarski, Adriana Zupa-Fernandez, Jeffrey A. Tredup, Kristen Pike, ChiehYing Chang, Dianlin Xie, Lihong Cheng, Donna Pedicord, Jodi Muckelbauer, Stephen R. Johnson, Sophie Wu, Suzanne C. Edavettil, Yang Hong, Mark R. Witmer, Lisa L. Elkin, Yuval Blat, William J. Pitts, David S. Weinstein, and James R. Burke

From the Departments of Molecular Structure and Design, Immunosciences Biology, Protein Science, Leads Discovery and Optimization, and Discovery Chemistry, Bristol-Myers Squibb Research and Development, Princeton, New Jersey 08543 and the Department of Leads Discovery and Optimization, Bristol-Myers Squibb Research and Development, Wallingford, Connecticut 06492

Background: Interleukin-23 mediates pathobiology in many autoimmune disorders.

Results: A chemogenomics approach identified small molecule agents that block receptor-mediated activation or tyrosine kinase 2 (Tyk2) and downstream signaling. Compounds stabilize the pseudokinase domain of Tyk2.

Conclusion: Small molecule ligands of the Tyk2 pseudokinase domain stabilize an autoinhibitory interaction with the catalytic domain.

Significance: This work enables the discovery of selective therapeutics targeting Tyk2-dependent pathways critical in autoimmunity.

Inhibition of signal transduction downstream of the IL-23 receptor represents an intriguing approach to the treatment of autoimmunity. Using a chemogenomics approach marrying kinome-wide inhibitory profiles of a compound library with the cellular activity against an IL-23-stimulated transcriptional response in T lymphocytes, a class of inhibitors was identified that bind to and stabilize the pseudokinase domain of the Janus kinase tyrosine kinase 2 (Tyk2), resulting in blockade of receptor-mediated activation of the adjacent catalytic domain. These Tyk2 pseudokinase domain stabilizers were also shown to inhibit Tyk2-dependent signaling through the Type I interferon receptor but not Tyk2-independent signaling and transcriptional cellular assays, including stimulation through the receptors for IL-2 (JAK1- and JAK3-dependent) and thrombopoietin (JAK2-dependent), demonstrating the high functional selectivity of this approach. A crystal structure of the pseudokinase domain liganded with a representative example showed the compound bound to a site analogous to the ATP-binding site in catalytic kinases with features consistent with high ligand selectivity. The results support a model where the pseudokinase domain regulates activation of the catalytic domain by forming receptor-regulated inhibitory interactions. Tyk2 pseudokinase stabilizers, therefore, represent a novel approach to the design of potent and selective agents for the treatment of autoimmunity.

In several human autoimmune diseases, such as psoriasis, rheumatoid arthritis, Crohn’s disease, and multiple sclerosis, a key pathogenic role for T helper 17 (T\textsubscript{h17})\textsuperscript{2} cells in mediating inflammation and tissue injury has been shown (for a review, see Ref. 1). Targeting the expansion and action of pathogenic T\textsubscript{h17} cells or mediators produced by these cells, therefore, has garnered considerable interest as a strategy toward the discovery of novel therapeutic agents. Particularly intensive efforts have been directed toward the discovery of agents that target interleukin-23 (IL-23), a cytokine critical in the expansion and survival of pathogenic T\textsubscript{h17} cells as well as the induction of innate lymphoid cells in autoimmunity (2, 3). Blocking monoclonal antibodies directed against either the p19 subunit of IL-23 or the p40 subunit it shares with IL-12 are being investigated in autoimmune diseases with clinical benefit demonstrated in patients with psoriasis and Crohn’s disease (4, 5).

Small molecule therapeutics targeting the IL-23 receptor (IL-23R) pathway represent especially intriguing approaches to autoimmunity, not only because of target load limitations with anti-cytokine monoclonal antibody therapeutics (e.g. the need to deliver sufficient antibody in relation to the level of cytokine), but perhaps more importantly, small molecule therapeutics provide the opportunity to target tissues that would be difficult to target with large protein-based therapies. Indeed, anti-p40 monoclonal antibodies have failed to show benefit in patients with multiple sclerosis, possibly due to an inability of the antibody to penetrate the central nervous system (6, 7).
Tyk2 Pseudokinase Domain Stabilizers Block Signal Transduction

The Janus protein kinase (JAK) family members tyrosine kinase 2 (Tyk2) and JAK2 are critical in the signal transduction pathways downstream of the IL-23 receptor (8). However, potential therapeutic agents that inhibit JAK2 would also result in anemia and other untoward hematopoietic defects due to its critical role in receptor signaling downstream of the receptors for erythropoietin, thrombopoietin, and other growth factors (8, 9). Targeting Tyk2 represents a more intriguing approach, especially because Tyk2-deficient mice are protected from many models of experimental autoimmunity, including collagen-induced arthritis and experimental autoimmune encephalomyelitis (10, 11). However, due to the high homology between the active sites in JAK kinase catalytic domains, only modest progress has been reported in the design and identification of potent and highly selective inhibitors of the catalytic activity of Tyk2 as potential therapeutics (12, 13).

In an effort to discover alternative and potentially more tractable kinase targets (and lead inhibitors) in the signaling pathway downstream of IL-23R, the present report details a chemogenomics approach that ultimately led to the identification of potent and selective inhibitors. These molecules act on Tyk2 but not by binding to the catalytic domain of the kinase and inhibiting catalytic activity, which is the classical mode for protein kinase inhibitors. Instead, the compounds were shown to prevent the receptor-mediated activation of the Tyk2 catalytic domain (also known as the JH1 domain) as a consequence of compound binding to and stabilizing the adjacent pseudokinase domain (also known as the JH2 domain), so called because it is evolutionarily related to protein kinases but is not thought to support catalytic activity (14). The pseudokinase domains of JAK family kinases have previously been implicated to play an autoinhibitory role in regulating activation of the adjacent catalytic domains (15, 16), but the mechanism by which this occurs is poorly understood. The cellular, biophysical, and structural biology studies described herein detail the mechanism by which these compounds block Tyk2-mediated signaling and transcription of the IL-23R receptor. This work provides the first demonstration that downstream signal transduction can be inhibited by pharmacological modulation at the level of the pseudokinase domain, and the findings also suggest that the regulation of Tyk2 activation may be unique to this JAK family member. This discovery provides an especially promising and novel approach to the design of potent and selective therapeutics for the treatment of autoimmune diseases dependent on Tyk2 signaling pathways.

**EXPERIMENTAL PROCEDURES**

**High Throughput IL-23-stimulated Transcriptional Response Assay**—kit225 human T cells with stable integration of a firefly luciferase reporter gene under the control of the interferon-γ activation sequence (IFN1-GAS-Luc) were grown in RPMI containing l-glutamine, 10% FBS, 20 ng/ml recombinant IL-2 (BIOSOURCE, PHC0023), and Geneticin at 0.7 μg/ml (from 50 mg/ml stock Gibco-BRL, 10131-035). Prior to the assay, cells were washed three times in assay medium (phenol red-free RPMI, 10% heat-inactivated FBS, 1% penicillin/streptomycin) to remove IL-2 present in the growth medium and allowed to incubate overnight. Immediately prior to assay, test compounds were dispensed into assay plates from library source plates via acoustic dispensing, followed by the addition of 65,000 cells/well in a total volume of 30 μl (10 μm final compound; 0.5% DMSO). IL-23 (prepared in PBS with 0.1% BSA) was added at a final concentration of 0.02 μM and allowed to incubate for 5 h at 37 °C. After incubating, 25 μl of Bright-Glo (Promega Corp.) was added prior to imaging the luminescent signal on the ViewLux.

For the primary screen, test compounds were evaluated at a single concentration (10 μM). As an assessment of screen robustness, Z' values for each plate were calculated; only plates with Z' > 0.5 were evaluated further. Percentage inhibition for each compound was determined, and a hit cut-off for the primary screen was set at mean + 1 × S.D. of all results (~40% inhibition); hits were confirmed by retesting in triplicate in the primary assay at 10 μM. Confirmed hits were further evaluated in 10-point, half-log concentration response assays.

**Kinome Screening Panel**—Compounds were screened at a concentration of 1 μM in competition binding assays at Ambit Biosciences as described previously (27). The technology employs kinases either produced as fusions to T7 phage or expressed as fusions to NF-κB in HEK-293 cells and subsequently tagged with DNA for PCR detection. Competition with test compound for binding to resin-conjugated affinity ligands, as measured by quantitative PCR, was used to determine the potency of compounds against each kinase.

**Chemogenomics**—Using a collection of 21,851 compounds with kinome-wide profiles against the panel of 386 kinases (17), compounds were excluded from the chemogenomics analysis if any of the following criteria were met: observed JAK2 or JAK3 activity, defined as >35% control in Ambit ligand displacement assays (or IC₅₀ < 0.5 μM in internal enzymatic assays); ≥20 kinases inhibited in the kinase panel with potency >67% inhibition at 1 μM; or >67% inhibition against any of CDK2, CDK5, or CDK7 in the Ambit ligand displacement assays. Only 7,908 of the 21,314 compounds passed all of these criteria.

**Compounds**—Synthesis of Compound 1 ((R)-N-(1-(3-(8-methyl-5-(methylamino)-8H-imidazo[4,5-d]thiazolo[5,4-b]pyridin-2-yl)phenyl)ethyl)-2-(methylsulfonyl)benzamide) was as follows. A solution of 2-bromo-5-chloro-8-methyl-5-(methylamino)-8H-imidazo[4,5-d]thiazolo[5,4-b]pyridin-2-yl)phenyl)ethylcarbamate (150 mg, 51% yield) as a solid. After cooling, the reaction mixture was filtered through a plug of silica gel and eluted with EtOAc/CH₂Cl₂ (3:1) to give the pure product (80 mg, 0.18 mmol) as a yellow solid.
Tyk2 Pseudokinase Domain Stabilizers Block Signal Transduction

A suspension that was heated by microwave at 150 °C for 30 min. The resulting dark brown solution was filtered through a PTFE filter and purified by reverse phase preparative HPLC to give (R)-tert-butyl 1-(3-(8-methyl-5-(methylamino)-8H-imidazo[4,5-d]thiazolo[5,4-b]pyridin-2-yl)phenyl)ethylcarbamate (75 mg, 76% yield) as a white solid. Hydrochloric acid (2 mL in EtOH, 0.8 μL, 1.6 mmol) was then added in one portion to the product, and the resulting heterogeneous mixture was stirred at room temperature for 12 h before removing the solvent under reduced pressure. The residue was dried under vacuum to give (R)-2-(3-(1-aminoethyl)phenyl)-N,8-dimethyl-8H-imidazo[4,5-d]thiazolo[5,4-b]pyridin-5-amine (50 mg, 93% yield). To a solution of this product from the last step (50 mg, 0.148 mmol) in N,N-dimethylformamide (1 mL) was added (1H-benzoazol-1-yl)oxo(8H-imidazo[4,5a]pyrazin-5-yl)phosphonium hexafluorophosphate (78 mg, 0.177 mmol), bisopropyl ethylamine (0.077 mL, 0.443 mmol), and 2-(methylsulfonyl)benzoic acid (44.4 mg, 0.222 mmol). The resulting mixture was stirred at room temperature for 5 h. After passing through a syringe tip PTFE filter, the reaction mixture was purified by reverse phase preparative HPLC to give Compound 1 (22 mg, 23% yield) as a white powder. 1H NMR (400 MHz, CDC13) δ ppm 8.08 (1 H, d, J = 2.0 Hz), 7.86 (1 H, d, J = 7.7 Hz), 7.60–7.78 (4 H, m), 7.51 (2 H, t, J = 7.7 Hz), 5.45 (1 H, q), 4.32 (3 H, s), 3.37–3.45 (3 H, m), 3.28 (3 H, br s), 1.75 (3 H, d, J = 6.8 Hz), MS (ES') m/z: 521 (MH+).

[1H]Compound 1 ([R]-N-(1-(3-(8-methyl-5-(methylamino)-8H-imidazo[4,5-d]thiazolo[5,4-b]pyridin-2-yl)phenyl)ethyl)cyanidido[methylsulfonyl]benzamide) was prepared as follows. 2-Mercaptobenzoic acid (2.3 mg, 0.015 mmol) and cesium carbonate (2 mg, 0.006 mmol) were added to a 5 mL round-bottomed flask. The flask was attached to a ported glass vacuum line, and the solution was mixed simultaneously. An ampoule of tritiated water (5 mL in EtOH, 0.8 mL) was added to the reaction flask, and stirring was maintained at room temperature for 4 h. The crude reaction mixture was purified by semipreparative HPLC to yield a total of 18 mCi of the desired product in 99.9% radiochemical purity. Mass spectral analysis of the tritiated product (m/z M + H 527.33) was used to establish the specific activity at 81 Ci/mmol.

The synthesis of BMS-066 has been reported previously (20). MLN120B was obtained from ChemScene (Monmouth Junction, NJ).

Signaling Assays in kit225 Cells and Peripheral Blood T Cells—STAT3 phosphorylation in kit225 cells induced by 25 ng/ml IL-23 (R&D Systems, 1290) was determined by ELISA (Cell Signaling, 7146) after a 20-min stimulation followed by the addition of lysis buffer (Cell Signaling) containing protease and phosphatase inhibitors. Activation of Tyk2 was determined in the same cells by measuring phosphorylation of Tyr-1054 and Tyr-1055 using an antibody that specifically recognizes Tyk2 phosphorylated at these sites (BD Biosciences). Briefly, cells were stimulated with 50 ng/ml IL-23 for 10 min, lysed, analyzed by SDS-PAGE (10% BisTris 1.0-mm gel, NuPage WG1201), and transferred to nitrocellulose membranes. Immunoblotting and detection with AlexaFluor680 goat anti-mouse IgG (Invitrogen, A21057) was performed, and the blots were analyzed using the LI-COR Odyssey scanner. The quantification of phosphorylated Tyk2 was normalized to actin, blotted simultaneously.

The effect of Compound 1 on IFNα- or IL-15-induced STAT phosphorylation was measured in human peripheral blood mononuclear cells stimulated with either 1000 units/ml IFNα (Peprotech, Rocky Hill, NJ) or 25 ng/ml IL-15 (Peprotech) for 15 min. Reactions were stopped by BD Phosflow lyse/fx buffer (BD Biosciences) at 37 °C, and after staining with CD3 fluorescein isothiocyanate-conjugated antibody, pSTAT1 PerCP-Cy5.5 (Tyr(P)-701), pSTAT3 PE (Tyr(P)-705), and pSTAT5 (Tyr(P)694 Alexa647-conjugated) antibodies, flow cytometry was used to gate on CD3+ T cells, and the amount of STAT phosphorylation was determined. Samples were acquired on a FACSCanto II using DIVA 6.1.1 software (BD Biosciences) and analyzed using FlowJo Vx (Tree Star). All conjugated antibodies were from BD Biosciences. Measurements of thrombopoietin-induced phosphorylation of STAT5 in platelets from human peripheral blood were performed as described previously (21).

Expression and Purification of Tyk2 Pseudokinase Domain—The coding region of the human Tyk2 pseudokinase domain (residues 575–869) was generated by PCR and cloned as an NdeI-XhoI fragment into a modified pFastBac1 vector (Invitrogen) with an N-terminal His-TMV tag to generate His-TMV-HTyk2 pseudokinase domain (residues 575–869)-pFB. The DNA sequence of the PCR product was sequence-verified. The TMV cleavage sequence is closely related to the TEV cleavage site, and the TMV viral protease recognizes the seven-residue sequence (ETVRFQ D G) with high selectivity, which leaves a single additional residue at the N terminus of the cleaved protein.

Baculovirus was generated for the His-TMV-HTyk2 pseudokinase domain (residues 575–869) construct using the Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer’s protocol. For large scale protein production, SF9 cells (Expression Systems, Davis, CA) grown in ESF921...
insect medium (Expression Systems) at 2 × 10^6 cells/ml were infected with virus stock at a 1:100 virus/cell ratio for 66 h. The production was carried out at 20-liter scale in a 50-liter Cellbag using the WAVE-Bioreactor system 20/50 (GE Healthcare). The infected cells were harvested by centrifugation, and wet insect cell pellet was dissolved at a 2.5:1 mass ratio of Buffer A (50 mM Hepes, pH 7.7, 500 mM NaCl, 25 mM imidazole, 5% (v/v) glycerol, 0.1% Triton X-100, and 0.5 mM TCEP), supplemented with protease inhibitor tablets (Roche Applied Science, catalog no. 13514100) and Benzonase (Sigma, catalog no. E8263-25KU). The cells were lysed by sonication, and the lysate was clarified by centrifugation at 9,500 rpm (Thermo, F10S-6x500Y rotor) for 30 min at 4 °C. Purification steps were executed using an AKTA Explorer-100 system. The supernatant was applied to a nickel affinity column (5-ml HisTrap FF crude, GE Healthcare, code 17-5286-01) washed to baseline with 50 mM Hepes, pH 7.7, 500 mM NaCl, 5% (v/v) glycerol, and 0.5 mM TCEP and then eluted with 50 mM Hepes, pH 7.7, 500 mM NaCl, 350 mM Imidazole, 5% (v/v) glycerol, and 0.5 mM TCEP. Fractions containing the target protein were concentrated with Amicon Ultra-15 centrifuge units (Millipore, catalog no. UFC901096). After concentration, the protein was further purified by size exclusion chromatography (HiLoad Superdex 75 (16/60), GE Healthcare, code 17-1068-01) run in 50 mM Hepes, pH 7.7, 500 mM NaCl, 1 mM MnCl₂, 5% (v/v) glycerol, 0.5 mM TCEP. For biophysical and crystallization experiments, the N-terminal His tag was removed by overnight cleavage at 4 °C, using His-tobacco vein motting virus protease at a 1:10 mass ratio. The next day, the His-tobacco vein motting virus protease and cleaved hexahistidine tag were bound to a 5-ml HisTrap FF crude column while the processed Tyk2 pseudokinase domain (residues 575–869) protein passed to a 5-ml HisTrap FF crude column while the processed Tyk2 pseudokinase domain (residues 575–869) protein passed into either 50 mM Na₂HPO₄, pH 7.7, 500 mM NaCl, 5% (v/v) glycerol, and 5 mM DTT) for long term storage at 0–33.3 °C. The infected cells were harvested by centrifugation, and wet stock sample of the molecule, in which the concentration was established by NMR quantitation relative to an internal standard. Data were analyzed with customized software, fitting baselines and the midpoint of the thermal transition, which was reported as the Tm value, with mean and S.D. values from triplicates reported.

Binding of human Tyk2 pseudokinase domain (residues 575–869) to the small molecule ligand, Compound 1, was evaluated by isothermal titration calorimetry using a VP-ITC instrument (MicroCal/GEHC) thermally controlled at 25 °C. The protein was extensively dialyzed against fresh buffer (10 mM Na₂HPO₄, pH 7.7, 500 mM NaCl) at 4 °C prior to titration, and the dialysate was retained for preparing the small molecule solution. A stock solution of Compound 1 was prepared to ~10 mM in DMSO-d₆ and the concentration was measured by NMR and reference to an internal standard. A working solution of ~50 μM was prepared, by diluting the stock solution in the buffer dialysate to a final DMSO-d₆ concentration of 0.50% (v/v). Protein concentration was adjusted by dilution to ~5 μM and confirmed by absorbance readings based on the amino acid composition (ε_280 = 31,160 M⁻¹ cm⁻¹). A small matching volume (i.e. 0.50% (v/v)) of DMSO-d₆ was added to the sample before transferring into the sample cell to minimize DMSO sample mismatch. Titrations were carried out in triplicate, and control injections of buffer into protein and Compound 1 into blank buffer were performed, both of which showed minimal background heats. A stirring rate of 307 rpm was maintained in the experiments, with an initial 2-μl injection, followed by 15–24 injections that varied between 5 and 10 μl in volume, spaced every 180 s. These injection profiles produced a 1.5–3.0-fold excess of ligand/protein for complete definition of the binding isotherm. The manufacturer’s software was used to analyze and plot the data, fitting to a 1:1 binding model.

A binding assay measuring the binding of [³H]Compound 1 to the N-terminal His-tagged Tyk2 pseudokinase domain was developed. Assays were performed in 384-well plates with a final assay volume of 20 μl containing copper-polyvinyltoluene scintillation proximity assay beads (PerkinElmer Life Sciences, catalog no. RPNQ0095) at 80 μg/ml, [³H]Compound 1 (20 nM), the N-terminal His-tagged Tyk2 pseudokinase domain (2.5 nM), and test compounds in assay buffer (50 mM HEPES, pH 7.5, 100 μg/ml BSA, 5% DMSO). After incubating at room temperature for 30 min, the inhibition was calculated by the displacement of [³H]Compound 1 binding as determined by scintillation counting. Dose-response curves were generated to determine the concentration required to inhibit [³H]-Compound 1 binding by 50% (IC₅₀).

Crystallization, Data Collection, and Structure Determination of Tyk2 Pseudokinase Domain in Complex with BMS-066—The Tyk2 pseudokinase domain (residues 575–869) at 1.2 mg/ml in 10 mM sodium phosphate, pH 7.7, 500 mM NaCl, 5% glycerol, 5 mM DTT was complexed with a 3-fold molar excess of ligand/protein for complete definition of the binding isotherm. Macroseeding was performed to obtain diffraction
size crystals. The crystals, grown at room temperature, began to appear in 1 week and took 2–3 weeks to reach full size (0.1 × 0.1 × 0.15 mm). Crystals were flash-cooled in liquid nitrogen with 25% glycerol and 75% reservoir solution as cryoprotectant. A 1.80 Å resolution data set was collected by Shamrock Structures, Inc. at Canadian Light Source beamline 08ID-1 with a Rayonix MX-300 detector. The data were processed and scaled using the program HKL2000 (22). The crystals had symmetry consistent with space group P1 with cell dimensions a = 41.5 Å, b = 63.5 Å, c = 68.0 Å (α = 74.4°, β = 75.5°, γ = 88.2°) and two molecules in the crystallographic asymmetric unit. The structure was determined by molecular replacement using the program Phaser (23) with an Abl kinase starting model (Protein Data Bank code 2GQG), where the residue side chains were stubbed, the activation loop and P-loop were removed, and the model was split into N-terminal (residues 243–319) and C-terminal (residues 320–500) domains. For molecular replacement, the two C-terminal domain orientations were found first, followed by the two N-terminal domains. Iterative cycles of model building and refinement using the programs reflac (24), autoBUSTER (25), and Coot (26) were carried out to build in side chain atoms and the missing loops. The model at this point was sufficient to clearly identify electron density for BMS-066. The location of the ligand was at the interface of the N- and C-terminal domains, near the hinge at residue Tyr-689 for both Tyk2 pseudokinase domain molecules. The final model of the Tyk2 pseudokinase domain consists of residues 580–609, 636–785, and 792–869 in molecule A and residues 580–609, 636–785, and 792–867 in molecule B (Tyk2 pseudokinase domain numbering), two ligand molecules, 25 sulfate ions, and 257 water molecules.

RESULTS

Chemogenomics Approach to Kinases Involved in IL-23R Signal Transduction—A library of 7,908 compounds selected from a collection of kinase inhibitors synthesized over the course of various kinase-targeted drug discovery efforts at Bristol-Myers Squibb throughout the last decade was used in a chemogenomics approach to identify inhibitors of IL-23R signal transduction in cells and elucidate their target mechanisms. The library was chosen for evaluation from a larger collection for which broad kinase inhibitory profiles were obtained at an inhibitor concentration of 1 μM against a panel of 386 unique human kinase and pseudokinase domains at Ambit Biosciences (hereafter referred to as the kinome screening panel), which employs competition binding assays to determine inhibitor potency (27). Because kinase inhibitors with broad inhibition across the kinome would confound the interpretation of the subsequent analysis of kinases involved in IL-23 signaling, compounds that were not reasonably selective (i.e. bind to more than 20 of 386 kinases in the panel) or likely to be simply cytotoxic to cells (e.g. cyclin-dependent kinase inhibitors) were excluded from the chemogenomics analysis. In addition, because JAK2 and Tyk2 were previously understood to be involved in IL-23R signal transduction, compounds were also excluded if they were known inhibitors of the enzymatic activity of these JAK family kinases.

Using kit225 human T cells into which a firefly luciferase reporter gene under the control of the interferon-γ activation sequence had been stably transfected, this library was evaluated at a concentration of 10 μM against IL-23-stimulated expression of the luciferase reporter gene. Hits showing >40% inhibition in the initial screen were confirmed by retesting in triplicate, with most confirmed hits evaluated in concentration response assays to calculate IC50 values.

With these cellular screening results, each kinase in the screening panel was evaluated for the “cell active rate” (CAR) defined as the percentage of compounds inhibiting a particular kinase by more than 90% at 1 μM that also inhibited the IL-23-stimulated reporter assay in kit225 T cells with an IC50 of <1 μM (or >75% inhibition at 10 μM if no IC50 had been generated). Associating cell activity with the percentage of inhibitors against a particular kinase allows for the potential identification of kinases regulating the cellular end point, even when using compounds that may not be exquisitely selective, and normalizes for the number of inhibitors of that particular kinase within the collection of compounds tested. A particular kinase is not necessarily important in regulating the IL-23 pathway based on the results with a promiscuous kinase inhibitor. Identifying inhibitors of that particular kinase with high cell active rate across the known inhibitors of that kinase, however, provides a more confident approach to identifying kinases truly involved in the pathway, but still requires additional follow up validation. As shown in Fig. 1A and supplemental Table S1, inhibitors of only three kinases in the screening panel yielded a corresponding CAR of greater than 10%. These include inhibitors of GSK-3α, IKKβ, and the pseudokinase (JH2) domain of Tyk2, which showed CAR values of 10.5, 12.2, and 15.6%, respectively. The identification of GSK-3α is not particularly surprising, because a role for GSK3 kinases in the regulation of STAT activation has been reported (28), although GSK3 kinases have not previously been implicated in IL-23R signal transduction specifically. The finding of a high CAR value for IKKβ, a critical regulator of canonical NF-κB activation, was not expected because the promoter upstream of the luciferase reporter in kit225 cells does not contain an NF-κB response element. Subsequent examination of the IKKβ inhibitors active against IL-23-induced luciferase expression in kit225 T cells revealed that the compounds represented imidazothiazolopyridines and related IKKβ inhibitors that also bind the pseudokinase domain of Tyk2. In other words, the apparent CAR for IKKβ and the Tyk2 pseudokinase domain result from a common set of compounds that bind to both proteins. Potent IKKβ inhibitors unrelated to this chemical series, such as MLN120B (29), do not bind to the Tyk2 pseudokinase domain and are inactive in the IL-23-stimulated kit225 T cell assay. These observations, along with additional evaluations on the mechanism of action (see below), indicate that the cellular activity of this set of compounds results from their action on the Tyk2 pseudokinase domain.

A representative example of this class of Tyk2 pseudokinase domain binders is Compound 1 (Fig. 1B) which, as shown in a representation of the kinome-wide profiling in Fig. 1C, is very potent against the Tyk2 pseudokinase (99% inhibition at 1 μM) but lacks potency against all other kinases in the kinome assay panel except IKKβ (96% inhibition) and the JAK1 pseudokinase domain (99% inhibition at 1 μM). No evidence of binding to the catalytic domain of Tyk2 or any other JAK family kinase was
evident at 1 M, and subsequent enzymatic assays confirmed the lack of activity against purified catalytic domains (IC\textsubscript{50} \geq 2 M). The pseudokinase domains of JAK2 and JAK3 were not part of the kinome screening panel. In the IL-23-stimulated kit225 T cell assay, Compound 1 inhibited the stimulated response with an IC\textsubscript{50} of 485 \pm 143 nM (n = 3). This figure is reproduced courtesy of Cell Signaling Technology, Inc.

Biophysical Characterization of the Binding of Compound 1 to the Pseudokinase Domain of Tyk2—The ability of Compound 1 to bind to the Tyk2 pseudokinase domain, as indicated by the kinome screening panel assay, was confirmed by isothermal titration calorimetry (ITC) and TSA. These experiments utilized a purified recombinant human protein comprising residues 575–869 of the Tyk2 pseudokinase domain. As shown in Fig. 2A, ITC measurements established that a high affinity binding interaction resulted from binding of the Tyk2 pseudokinase domain and Compound 1, with a K\textsubscript{D} value in the low nanomolar range. The mean value obtained from triplicate experiments was K\textsubscript{D} = 5 \pm 4 nM, approaching the limit of determination under the direct titration method used here. The experiments revealed an exothermic binding reaction, with a mean value of ΔH\textsubscript{obs} = −9.4 \pm 0.2 kcal/mol, reflecting favorable changes in binding enthalpy for the system, a value within a typical range for many small molecule-protein interactions (30). Because the titration was performed in phosphate buffer, which has ΔH\textsubscript{ion} of ~0 kcal/mol, the observed enthalpy, ΔH\textsubscript{obs}, approaches ΔH\textsubscript{bio} for this system. In other words, the observed enthalpy does not reflect significant changes from buffer protonation/deprotonation but instead reflects net changes in the bound versus free states of protein and ligand (31). Consequently, the majority of the free energy change (ΔG) of this
binding reaction reflects exothermic stabilization (ΔH) of the system. The method also yielded a stoichiometry for the interaction between Compound 1 and the Tyk2 pseudokinase domain of 0.5–0.7, modestly lower than 1.0. The data were interpreted as reflecting 1:1 binding, consistent with the x-ray structure (see below).

To determine the effect of Compound 1 binding on the Tyk2 pseudokinase domain, the thermal unfolding of protein was monitored by fluorescence spectroscopic measurements with SYPRO Orange. The fluorescence emission of this dye significantly increases as hydrophobic surfaces of globular proteins are exposed to the aqueous solution upon heat-induced denaturation. As shown in Fig. 2B, the apo-Tyk2 pseudokinase domain showed a midpoint on the protein stability curve (melting temperature, Tm) of 43.5 °C, and titration of Compound 1 with a 1–2-fold molar excess resulted in an increase in the Tm to >50 °C, with a maximum ΔTm = 14 °C at 8-fold more excess. This large positive ΔTm shift demonstrated that Compound 1 was bound to a region of the protein that manifests an increase in stability, although this method cannot be used to determine a KD value (32). Combining the results from both the ITC and TSA experiments, Compound 1 shows enthalpically driven, high affinity binding to the Tyk2 pseudokinase domain, which results in a pronounced stabilization of the pseudokinase domain. Recombinant, full-length Tyk2 is poorly behaved and could not be used in thermal shift assays.

Pharmacologic Stabilization of the Tyk2 Pseudokinase Inhibits Activation and STAT Signaling—Because the pseudokinase domain is thought to lack catalytic activity, precluding the use of an enzymatic activity assay to measure compound potency, a homogeneous ligand displacement assay was developed to evaluate the potency of additional analogs in concentration response determinations. Using a radiolabeled version of Compound 1 ([3H]Compound 1) as the ligand in a homogeneous binding assay using the Tyk2 pseudokinase domain complexed to scintillation proximity assay beads, a KD of ~10 nm was determined. As detailed in Fig. 3A, evaluation of compounds in this series as well as in chemically unrelated compounds showed that a strong correlation exists between potency against the IL-23-stimulated kit225 T cell assay and in the Tyk2 pseudokinase domain, as measured by displacement of [3H]Compound 1 (Spearman’s correlation coefficient = 0.8). As expected, there was no correlation between cellular and IKKβ potency (see Fig. 3B). A limitation of the present work is that an inhibitor-insensitive mutant form of Tyk2 is not available to further demonstrate that these compounds act through the Tyk2 pseudokinase domain.

In an effort to probe the mechanism by which Tyk2 pseudokinase domain stabilizers, such as Compound 1, inhibit IL-23-induced transcriptional responses, the effect on Tyk2-catalyzed phosphorylation of STAT3 was determined. As shown in Fig. 1D, Compound 1 inhibited IL-23-stimulated phosphorylation of STAT3 in kit225 T cells with a potency equipotent to that obtained against transcription of the STAT-responsive reporter gene in the same experiment, indicating that the Tyk2 pseudokinase domain stabilizer blocks Tyk2-catalyzed phosphorylation of STAT proteins even without directly interacting with the catalytic domain of Tyk2. Because phosphorylation of Tyr-1054 and Tyr-1055 within the activation loop of the catalytic domain of Tyk2 is critical in inducing a catalytically active form of the kinase (33), the effect of Compound 1 on Tyr-1054/Tyr-1055 phosphorylation upon receptor-mediated stimulation was investigated. Fig. 3C shows that Compound 1 inhibited the IL-23-stimulated phosphorylation of Tyr-1054/Tyr-1055 in a concentration-dependent manner, with a potency (IC50 ~500 nM) consistent with the effects on both STAT3 phosphorylation and STAT-dependent reporter gene expression in these cells.

Many of the cytokine and growth factor receptors that rely upon JAK kinases often utilize two members of the family to mediate downstream signal transduction, and JAK2 along with Tyk2 has been implicated in IL-23R signaling. In order
to determine the specificity for Tyk2-dependent functional signaling, Compound 1 was evaluated in human peripheral blood T cells for effects on the phosphorylation of STAT proteins using agonists of receptors both dependent on and independent of Tyk2. Type I interferon receptor signaling requires both Tyk2 and JAK1, and as shown in Fig. 3D, Compound 1 inhibited IFNα-induced phosphorylation of STAT1, STAT3, and STAT5 with equivalent potency (IC₅₀ ~500 nM). However, the compound was ineffective against signaling pathways that do not require Tyk2, as evidenced by the lack of activity against both thrombopoietin-stimulated phosphorylation of STAT5 (JAK2-dependent signaling) and IL-15-induced phosphorylation of STAT5 (JAK1- and JAK3-dependent signaling).

Because Compound 1 at 1 μM showed inhibition against the pseudokinase domain of JAK1 in the kinome screening panel nearly equal to that shown against the Tyk2 pseudokinase domain, the lack of functional activity against JAK1-dependent IL-15 receptor signaling is surprising. The results may suggest that the mechanism by which the pseudokinase domain of JAK1 regulates the catalytic domain differs from Tyk2 and that JAK1 pseudokinase domain binders cannot prevent the activation of kinase activity. Alternatively, it may be that the isolated recombinant JAK1 pseudokinase domain can obtain a conformation in vitro that accommodates binding of Compound 1, but the endogenous full-length multidomain protein present in cells cannot. Although we do not have well behaved full-length JAK1 (or Tyk2) recombinant proteins to more fully discriminate between these potential explanations for the observed difference in functional activity, it is important to note that more than 100 other compounds that appear to be JAK1 pseudokinase binders have been evaluated and failed to show functional potency in JAK1-dependent cellular assays (data not shown). This indicates that the effect is not unique to Compound 1. The pseudokinase domains of JAK2 and JAK3 have not been evaluated to determine whether Compound 1 can bind in vitro, but our data suggest that, at least for this class of Tyk2 pseudokinase binders, there is a high level of functional selectivity in cellular assays. As detailed below, the Tyk2 pseudokinase domain binding site for this class of compounds has unique features when compared with the pseudokinase domains of the other JAK family members, which may provide some rationale for the observed selectivity.
Crystal Structure of Tyk2 Pseudokinase Domain with Bound Stabilizer—To help understand the mechanism by which Compound 1 and related Tyk2 pseudokinase domain stabilizers prevent the activation of the catalytic domain and to aid future compound design efforts, we obtained crystal structures of the Tyk2 pseudokinase domain bound with compounds in this series. We were unsuccessful in obtaining a crystal structure of Tyk2 pseudokinase domain in complex with Compound 1; however, an x-ray crystal structure bound to a less potent analog, BMS-066 (see Fig. 1B), was determined to 1.8 Å resolution. This compound showed IC$_{50}$ values of 72 and 1020 nM against the Tyk2 pseudokinase domain probe displacement and IL-23-stimulated reporter assays, respectively (data not shown). As shown in Fig. 4A, the crystal structure of the Tyk2 pseudokinase domain encompasses the main structural features of a protein kinase. Namely, the protein fold is separated into two subdomains, or lobes, with the smaller N-terminal lobe composed of a five-stranded $\beta$ sheet and an additional short strand from residues 589–591 in the N terminus as well as the prominent helix $\alpha$-C. The C-terminal lobe is larger than the N-terminal lobe and is predominantly helical. The two lobes are connected through a single polypeptide strand (the linker/hinge region), which in catalytic kinases is normally involved in binding to the adenine ring of ATP. The site analogous to the ATP-binding site in catalytic kinases forms a deep cleft between the two lobes and sits beneath the phosphate binding loop (P-loop) connecting strands $\beta$1 and $\beta$2. BMS-066 with magenta carbons is shown in a stick representation.
Tyk2 Pseudokinase Domain Stabilizers Block Signal Transduction

Consistent with this domain being defined as a pseudokinase, the crystal structure shows a number of amino acid and structural differences that, when compared with catalytic kinases, indicate that this domain is unable to support catalytic activity. For example, the typically conserved β3-strand lysine (Lys-642) is present but does not form a salt bridge with a conserved glutamate from helix α-C (important for maintaining an active conformation in catalytic kinases). Instead, Thr-658 occupies the position of the typical conserved glutamate. The preceding residue in the helix is interestingly Glu-657, but it points toward solvent (see Fig. 4B). Lys-642 in the pseudokinase domain structure is instead near hydrogen bonding distance to Asp-759, the aspartate that in catalytic kinases belongs to the conserved DFG motif, which marks the N-terminal portion of the activation loop and normally interacts with the ATP phosphates through Mg^{2+}. In the Tyk2 pseudokinase domain, this motif is replaced by DPG, whereby proline appears to restrict the position of Asp-759 to a region normally occupied by the helix α-C glutamate. The altered position of Lys-642 and Asp-759 would not optimally interact with Mg^{2+}-ATP. Moreover, the P-loop that normally serves as a gate to ATP binding in catalytic protein kinases usually contains a conserved glycine-rich sequence motif (GXGXXG), where ϕ, usually tyrosine or phenylalanine, caps the site of phosphate transfer, but in the Tyk2 pseudokinase domain the sequence is GXGXT, which might be expected to reduce the flexibility of the P-loop due to the loss of the glycine and the rigidifying effects of the hydrogen bonding interactions of the P-loop Arg-600 (see below). A sulfate ion (a crystallization solution ingredient) is observed under the P-loop and is also involved in hydrogen bonds to the side chains of the P-loop Thr-599 and Thr-601 and the backbone NH of Thr-599. The activation loop, which in catalytic kinases not only provides the binding platform for peptide substrate interactions but also ensures the appropriate orientation of the catalytic machinery, is truncated compared with the catalytic domain activation loop (20 residues versus 26) and adopts a partially helical conformation. Interactions that appear to stitch the N-terminal lobe and the C-terminal lobe together to hinder ATP and substrate binding include a salt bridge between the P-loop Arg-600 and the activation loop Glu-771 (both conserved only in the JAK1 pseudokinase domain) and a hydrogen bond between Arg-738 and the P-loop carbonyl of Gln-597 through a water (Fig. 4C). Perhaps the most important difference compared with catalytic kinases is that the canonical protein kinase catalytic loop motif HRD (D is the catalytic aspartate that contributes to the nucleophilicity of the substrate hydroxyl) is replaced by HGN (Asn-734) in the Tyk2 pseudokinase domain. Asn-734 is observed to be involved in a hydrogen bond with the backbone carbonyl of Pro-760 of the DPG motif (Fig. 4C). Interestingly, the surface of the protein near the activation loop, usually the site of substrate binding, is rather concave in nature (see Fig. 4D), which may indicate that it could be involved in protein-protein interactions. In summary, the particular conformation of the activation loop, the interactions between the P-loop and C-terminal lobe (particularly the activation loop), and the absence of the catalytic aspartate do not appear to be consistent with an ability of the domain to bind either ATP or peptide substrate and catalyze phosphoryl transfer as an active kinase.

BMS-066 sits in the deep cleft between the N- and C-terminal lobes, with the tricyclic ring system of BMS-066 occupying the site typically bound by the adenine group of ATP in catalytic kinases (see Fig. 5A). The imidazole basic nitrogen and the amino moiety form hydrogen bonds with Val-690 of the hinge. The imidazole N-methyl group sits in a hydrophobic pocket composed of β3-strand Val-640 and β6-strand Leu-741, and the pendant pyridine ring is sandwiched between the β1-strand of the P-loop and Pro-694 and Arg-738 of the extended hinge. The amide NH hydrogen of the methoxyacetamide group hydrogen bonds with the backbone carbonyl of the P-loop Leu-595 as the group protrudes out toward solvent. A water molecule deep in the pocket forms hydrogen bonds with the side chain of the gatekeeper Thr-687 and the hinge carbonyl from Glu-688, and there is also a water hydrogen bond with the pyridine nitrogen of the tricycle. Docking of Compound 1 into the solved Tyk2 pseudokinase domain structure suggests that the hinge-binding interactions are identical to BMS-066 but that the rest of the molecule may bind deeper into the binding site, burying more surface area and possibly making a number of additional hydrogen bonds, which might explain its enhanced potency. A potential binding model is provided in Fig. 6.

Recently, structures of the Tyk2 pseudokinase domain and the Tyk2 pseudokinase-kinase dual domain were released in the Protein Data Bank (PDB entries 3ZON and 4OLI, respectively (34). An α-carbon backbone alignment of the structure described here and the released versions reveals that the Tyk2 pseudokinase domain structures adopt very similar conformations (PDB entry 3ZON, root mean square deviation 0.7 Å; PDB entry 4OLI, root mean square deviation 0.8 Å). Comparison of the overall fold of the Tyk2 pseudokinase domain structure with recently published JAK1 and JAK2 JH2 structures (35, 36) also shows high similarity (Fig. 5B).

Not only are there differences in the Tyk2 pseudokinase domain from catalytic kinases related to its inability to function as a kinase, but a sequence comparison of the BMS-066 binding site residues with the 491 sequences of the kinome provides the basis for high selectivity of a Tyk2 pseudokinase domain stabilizer. Besides the difference in orientation of the conserved Lys-642 and Asp-759 of DPG compared with a protein kinase in the active state, there are a number of other uncommon residues near the BMS-066 binding site. As shown in Fig. 5C, these differences include Pro-760 of the DPG motif, which is not present within any other kinase. In addition, the gatekeeper residue is a Thr (Thr-687, 19% prevalence in kinome), the residue below the gatekeeper is an Ala (Ala-671, 2% in kinome), and the residue before DPG is a Ser (Ser-758, 15% kinome), with the combination of the three being unique to the kinome and indicating that the pocket is well suited to design highly selective therapeutics. In the extended hinge, Pro-694 is found in only about 2% of the kinome, and the nearby Val-697 is found in less than 1% of the kinome. In addition, the binding site residues within the JAK JH2 family are not highly conserved, presenting an opportunity to design pseudokinase domain selectivity as well (Fig. 7A).
DISCUSSION

Typically, kinase inhibitors have been targeted to the ATP binding site, and although this approach has been successful in identifying potent inhibitors, the design of therapeutic agents with selectivity across a target class of more than 500 human protein kinases can be quite challenging due to the highly conserved nature of this binding site. Selectivity has proven to be especially difficult to obtain within the JAK family kinases, which have highly conserved active sites. Targeting the pseudokinase domain of Tyk2, as identified using the chemogenomics strategy detailed in the present report, represents a novel approach in the design of highly selective inhibitors of Tyk2-dependent signaling downstream of the IL-23 receptor and other pathways important in autoimmunity. Although it had been previously understood that the pseudokinase domains of Tyk2 and the other JAK family kinases play a critical role in the regulation of receptor-mediated activation of the catalytic domain, Compound 1 and related imidazothiazolopyridines...
Tyk2 Pseudokinase Domain Stabilizers Block Signal Transduction

Although the exact molecular mechanism by which the pseudokinase domain of Tyk2 regulates receptor-mediated activation of the catalytic domain is not known, much of the published data support a model where the pseudokinase domain normally forms an intramolecular interaction with the catalytic domain such that the pseudokinase domain either induces an inactive conformation of the catalytic domain, or the pseudokinase domain blocks access of substrates or ATP by a direct interaction in this region (34, 36, 39, 40). A putative receptor-mediated conformational change in the pseudokinase domain would then break the intramolecular autoinhibitory interaction with the catalytic domain, allowing the catalytic domain to achieve a conformation that allows for both phosphorylation of the activation loop and catalytic activity. Studies of the β5-strand Val to Phe mutations in the JH2/pseudokinase domains of JAK1, JAK2, and Tyk2, all of which lead to constitutive activity (34, 42, 43, 44), are consistent with this model. In the case of JAK1, structural analysis of the JAK1 pseudokinase domain WT and V658F mutant domains showed the mutant V658F packs in an edge-to-face manner with α-C helix Phe-636 and fits in the same position occupied by Phe-575 of the SH2-PK linker in the wild-type structure. Therefore, the V658F mutation induces a coordinated rearrangement of the SH2-PK linker. Interestingly, one of the monomers in the asymmetric unit of the wild type JAK1 pseudokinase domain structure also displays this rearrangement, which has been proposed to be a structural switch controlling catalytic activation. The residues involved in rearrangement in JAK1 and JAK2 are conserved in the Tyk2 pseudokinase domain except for α-C helix Phe (JAK2 Phe-595), which is conservatively replaced by a Tyr (Fig. 7B). Based on our current understanding, the mechanism of catalytic domain regulation by the pseudokinase domain involving α-C helix and the SH2-PK linker interactions in JAK1 are probably similar for Tyk2. It is therefore both surprising and intriguing that Compound 1 is selective for receptor-mediated pathways dependent on Tyk2 compared with those receptors dependent on other JAK family members because the compound binds to the pseudokinase domain of JAK1 but does not impact JAK1-dependent (but Tyk2-independent) receptor signaling. The compound also failed to block JAK2- and JAK3-dependent signaling. The selectivity for Tyk2-dependent signaling may reflect a mechanistic attribute of Tyk2 that is unique, or the apparent binding to the JAK1 pseudokinase domain is artificial. An increased

represent the first demonstration that this mechanism can be targeted by small molecule agents to block receptor-mediated signaling. That pseudokinase stabilizers block the activation of the kinase domain is especially relevant to autoimmunity because a coding variant of Tyk2 resulting in a change from Pro-1104 to alanine has been shown to similarly block activation of the catalytic domain (37), and individuals carrying this allele are protected from multiple sclerosis and possibly other autoimmune disorders (37, 38).

Detailed work with JAK1 is consistent with the pseudokinase domain forming an inhibitory interaction with the catalytic domain (36), and a model of this intramolecular autoinhibitory interaction between pseudokinase domain and catalytic domains of JAK kinases postulates that one of the contact surfaces includes an α-C helix-α-C helix interface, and another one includes the catalytic domain activation loop, where the latter would be precluded from adopting an active conformation (41). Additional evidence of an intramolecular autoinhibitory interaction mediated by the pseudokinase domain of Tyk2 was recently provided by a crystal structure of a dual domain pseudokinase-kinase construct, with the interaction appearing to limit the conformational mobility of the catalytic domain active site (34). Upon a receptor-induced conformational change in the pseudokinase domain mediated via the FERM domain and SH2-pseudokinase (SH2-PK) linker, the intramolecular autoinhibitory interaction with the catalytic domain is broken, allowing the catalytic domain to achieve a conformation that allows for both phosphorylation of the activation loop and catalytic activity. Studies of the β5-strand Val to Phe mutations in the JH2/pseudokinase domains of JAK1, JAK2, and Tyk2, all of which lead to constitutive activity (34, 42, 43, 44), are consistent with this model. In the case of JAK1, structural analysis of the JAK1 pseudokinase domain WT and V658F mutant domains showed the mutant V658F packs in an edge-to-face manner with α-C helix Phe-636 and fits in the same position occupied by Phe-575 of the SH2-PK linker in the wild-type structure. Therefore, the V658F mutation induces a coordinated rearrangement of the SH2-PK linker. Interestingly, one of the monomers in the asymmetric unit of the wild type JAK1 pseudokinase domain structure also displays this rearrangement, which has been proposed to be a structural switch controlling catalytic activation. The residues involved in rearrangement in JAK1 and JAK2 are conserved in the Tyk2 pseudokinase domain except for α-C helix Phe (JAK2 Phe-595), which is conservatively replaced by a Tyr (Fig. 7B). Based on our current understanding, the mechanism of catalytic domain regulation by the pseudokinase domain involving α-C helix and the SH2-PK linker interactions in JAK1 are probably similar for Tyk2. It is therefore both surprising and intriguing that Compound 1 is selective for receptor-mediated pathways dependent on Tyk2 compared with those receptors dependent on other JAK family members because the compound binds to the pseudokinase domain of JAK1 but does not impact JAK1-dependent (but Tyk2-independent) receptor signaling. The compound also failed to block JAK2- and JAK3-dependent signaling. The selectivity for Tyk2-dependent signaling may reflect a mechanistic attribute of Tyk2 that is unique, or the apparent binding to the JAK1 pseudokinase domain is artificial. An increased

sponding residue in Tyk2 is Pro-694, which would appear to sterically clash with the JAK2-bound ATP. We have been unable, furthermore, to demonstrate that ATP or non-hydrolyzable ATP analogs can bind to the Tyk2 pseudokinase domain, either through the use of biophysical methods, such as thermal shift assays, or by displacement of radiolabeled Compound 1 binding to the Tyk2 pseudokinase domain (results not shown).
Tyk2 Pseudokinase Domain Stabilizers Block Signal Transduction

Tyk2 Pseudokinase Domain Stabilizers Block Signal Transduction

A

B

FIGURE 7. Unique features of pseudokinase domain of Tyk2 compared with other JAK members. A, Tyk2 pseudokinase domain structure bound with BMS-066 highlighting residues that are different compared with a representation in the JAK pseudokinase domain family. B, superposition of Tyk2 pseudokinase domain/BMS-066 structure (green) with JAK1 pseudokinase domain (magenta; PDB code 4L00) and JAK2 JH2 (yellow; PDB code 4FVQ), highlighting residues that may be involved in stabilization and triggering release of the autoinhibitory hold of the pseudokinase domain over the kinase domain.

understanding of JAK family kinase regulation will be required to fully account for these observations.

In summary, the present results are consistent with a model in which small molecule ligands of the Tyk2 pseudokinase domain act to lock it in a conformation that stabilizes an autoinhibitory interaction with the catalytic domain. This stabilization prevents receptor-mediated activation and/or catalytic activity of the catalytic domain, blocking downstream signal transduction and STAT-dependent gene transcription. Targeting the pseudokinase domain of Tyk2 represents a novel approach in the design of highly selective therapeutics targeting Tyk2-dependent signaling important in autoimmunity. While additional investigations into both the mechanisms by which Tyk2 pseudokinase domain stabilizers block the activation of the catalytic domain and the unique attributes of Tyk2 compared with other JAK family members are underway, we are capitalizing on this finding to optimize potent and selective agents for the treatment of human autoimmune diseases. Detailed evaluations of the pharmacology of selective Tyk2 pseudokinase stabilizers will be the subject of a future report.

Acknowledgments—We thank Greg Ford for providing the IL-23-responsive luciferase reporter cells; Joann Strnad and Mian Gao for contributions to the synthesis of Compound 1 and BMS-066.

REFERENCES

1. Miossec P., and Kolls, J.K. (2012) Targeting IL-17 and TH17 cells in chronic inflammation. Nat. Rev. Drug Discov. 11, 763–776
2. Volpe, E., Servant, N., Zollinger, R., Bogiatzi, S. I., Hupé, P., Barillot, E., and Soumelis, V. (2008) A critical function for transforming growth factor-β, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. Nat. Immunol. 9, 650–657
3. Geremia, A., Arancibia-Cárcamo, C. V., Fleming, M. P., Rust, N., Singh, B., Mortensen, N. I., Travis, S. P., and Powrie, F. (2011) IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease. J. Exp. Med. 208, 1127–1133
4. Sandborn, W. J., Gasink, C., Gao, L. L., Blank, M. A., Johanss, J., Guzzo, C., Sands, B. E., Hanauer, S. B., Targan, S., Rutgeerts, P., Ghosh, S., de Villiers, W. J., Panaccione, R., Greenberg, G., Scheirer, S., Lichtiger, S., Feagan, B. G., and CERTIFI Study Group (2012) Ustekinumab induction and maintenance therapy in refractory Crohn’s disease. N. Engl. J. Med. 367, 1519–1528
5. Strober, B. E., Crowley, J. J., Yamauchi, P. S., Olds, M., and Williams D. A. (2011) Efficacy and safety results from a phase III, randomized controlled trial comparing the safety and efficacy of briakinumab with etanercept and placebo in patients with moderate to severe chronic plaque psoriasis. Br. J. Dermatol. 165, 661–668
6. Longbrake, E. E., and Racke, M. K. (2009) Why did IL-12/IL-23 antibody therapy fail in multiple sclerosis? Expert Rev. Neurother. 9, 319–321
7. Vollmer, T. L., Wynn, D. R., Alam, M. S., and Valdes, J. (2011) A phase 2, 24-week, randomized, placebo-controlled, double-blind study examining the efficacy and safety of an anti-interleukin-12 and -23 monoclonal antibody in patients with relapsing-remitting or secondary progressive multiple sclerosis. Mult. Scler. 17, 181–191
8. Murray, P. J. (2007) The JAK-STAT signaling pathway: input and output integration. J. Immunol. 178, 2623–2629
9. Vincenti, F., Tedesco Silva, H., Busque, S., O’Connell, P., Friedewald, J., Cibrik, D., Budde, K., Yoshida, A., Coheyn, S., Weimar, W., Kim, Y. S., Lawndy, N., Lan, S. P., Kudlacz, E., Krishnaswami, S., and Chan, G. (2012) Randomized phase 2b trial of tofacitinib (CP-690,550) in de novo kidney transplant patients: efficacy, renal function and safety at 1 year. Am. J. Transplant. 12, 2446–2456
10. Oyamada, A., Ikebe, H., Itsuini, M., Saiwai, H., Okada, S., Shimoda, K., Iwakura, Y., Nakayama, K. I., Iwamoto, Y., Yoshikai, Y., and Yamada, H. (2009) Tyrosine kinase 2 plays critical roles in the pathogenic CD4 T cell responses for the development of experimental autoimmune encephalomyelitis. J. Immunol. 183, 7539–7546
11. Ortman, R., Smeltz, R., Yap, G., Sher, A., and Shevach, E. M. (2001) A heritable defect in IL-12 signaling in B10.Q/J mice. I. In vitro analysis. J. Immunol. 166, 5712–5719
12. Liang, J., van Abbema, A., Balazs, M., Barrett, K., Berezhkovsky, L., Blair, W., Chang, C., Delarosa, D., DeVoss, J., Driscoll, J., Eigenbrot, C., Ghilardi, N., Gibbons, P., Halladay, J., Johnson, A., Kohli, P. B., Lai, Y., Liu, Y., Lyssikatos, J., Mantik, P., Menghradjani, K., Murray, J., Peng, I., Sambrone, A., Shia, S., Shin, Y., Smith, J., Sohn, S., Tsui, V., Uttsch, M., Wu, L. C., Xiao,
Tyk2 Pseudokinase Domain Stabilizers Block Signal Transduction

Y., Yang, W., Young, I., Zhang, B., Zhu, B. Y., and Magnussen, S. (2013) Lead optimization of a 4-aminoypyridine benzamide scaffold to identify potent, selective, and orally bioavailable TYK2 inhibitors. *J. Med. Chem.* 56, 4521–4536

13. Norman, P. (2012) Selective JAK1 inhibitor and selective Tyk2 inhibitor patents. *Expert Opin. Ther. Pat.* 22, 1233–1249

14. Zeqiraj E., and van Aalten, D.M. (2010) Pseudokinases-remnants of evolutionary key allosteric regulators? *Curr. Opin. Struct. Biol.* 20, 772–781

15. Saharinen, P., Vihinen, M., and Silvennoinen, O. (2003) Autoinhibition of Jak2 tyrosine kinase is dependent on specific regions in its pseudokinase domain. *Mol. Biol. Cell.* 14, 1448–1459

16. Saharinen, P., and Silvennoinen, O. (2002) The pseudokinase domain is required for suppression of basal activity of Jak2 and Jak3 tyrosine kinases and for cytokine-inducible activation of signal transduction. *J. Biol. Chem.* 277, 47954–47963

17. Posy, S. L., Herrmeier, M. A., Vaccaro, W., Ott, K. H., Todderud, G., Lippy, J. S., Trainor, G. L., Loughnay, D. A., Johnson, S. R. (2011) Trends in kinase selectivity: insights for target class-focused library screening. *J. Med. Chem.* 54, 54–66

18. Kempson, J., Spergel, S. H., Guo, J., Quesnelle, C., Gill, P., Belanger, D., Posy, S. L., Hermsmeier, M. A., Vaccaro, W., Ott, K. H., Todderud, G., Zeqiraj E., and van Aalten, D.M. (2010) Pseudokinases-remnants of evolutionary key allosteric regulators? *Curr. Opin. Struct. Biol.* 20, 772–781

19. Norman, P. (2012) Selective JAK1 inhibitor and selective Tyk2 inhibitor patents. *Expert Opin. Ther. Pat.* 22, 1233–1249

20. Gillooly, K. M., Pattoli, M. A., Booth-Lute, H., Yang, G., Davies, P., Kukral, D. W., Strnad, J., McIntyre, J., Dwyer, K. W., Dario, C. J., Sailer-Cid, L., Yang, Z., Wang-Iverson, D., Dodd, J. H., McKinnon, M., Barrish, J. C., and Pitts, W. J. (2009) Novel tricyclic inhibitors of JAK kinase selectivity: insights for target class-focused library screening. *J. Med. Chem.* 52, 1046–1051

21. Purandare, A. V., McDevitt, T. M., Wan, H., You, D., Penhallow, B., Han, Yang, Z., Marathe, P. H., Wang-Iverson, D., Dodd, J. H., McKinnon, M., Barrish, J. C., and Pitts, W. J. (2009) Novel tricyclic inhibitors of JAK kinase selectivity: insights for target class-focused library screening. *J. Med. Chem.* 52, 1046–1051

22. Booth-Lute, H., Yang, G., Davies, P., Kukral, D. W., Strnad, J., McIntyre, J., Dwyer, K. W., Dario, C. J., Sailer-Cid, L., Yang, Z., Wang-Iverson, D., Dodd, J. H., McKinnon, M., Barrish, J. C., and Pitts, W. J. (2009) Novel tricyclic inhibitors of JAK kinase selectivity: insights for target class-focused library screening. *J. Med. Chem.* 52, 1046–1051

23. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Sturrock, R. F., Terwilliger, T. C., and Read, R. J. (2007) Phaser crystallographic software. *Acta Crystallogr. D Biol. Crystallogr.* 63, 1205–1212

24. Bricogne, G., Blanc, E., Brandl, M., Flensburg, C., Keller, P., Paciorek, W., Pieri, L., Bogani, C., Guglielmelli, P., Zingariello, M., Rana, R. A., Bartulucci, N. P., Duddy, B., Waringer, S., Hauser, S. L., Sexton, D., Haines, J., Sawyer, S., Wellcome Trust Case-Control Consortium (WTCCC), and Compston, A. (2009) Replication analysis identifies TYK2 as a multiple sclerosis susceptibility factor. *Eur. J. Hum. Genet.* 17, 1309–1313

25. Bricogne, G., Blanc, E., Brandl, M., Flensburg, C., Keller, P., Paciorek, W., Rana, R. A., Bartulucci, N. P., Duddy, B., Waringer, S., Hauser, S. L., Sexton, D., Haines, J., Sawyer, S., Wellcome Trust Case-Control Consortium (WTCCC), and Compston, A. (2009) Replication analysis identifies TYK2 as a multiple sclerosis susceptibility factor. *Eur. J. Hum. Genet.* 17, 1309–1313

26. Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010) Features and for the refinement of macromolecular crystal structures. *Acta Crystallogr. D Biol. Crystallogr.* 66, 283–288

27. Fersht, A. R. (1994) The secondary structure and stability of proteins in solution. *J. Mol. Biol.* 235, 783–804

28. Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010) Features and for the refinement of macromolecular crystal structures. *Acta Crystallogr. D Biol. Crystallogr.* 66, 283–288
Tyrosine Kinase 2-mediated Signal Transduction in T Lymphocytes Is Blocked by Pharmacological Stabilization of Its Pseudokinase Domain

John S. Tokarski, Adriana Zupa-Fernandez, Jeffrey A. Tredup, Kristen Pike, ChiehYing Chang, Dianlin Xie, Lihong Cheng, Donna Pedicord, Jodi Muckelbauer, Stephen R. Johnson, Sophie Wu, Suzanne C. Edavettal, Yang Hong, Mark R. Witmer, Lisa L. Elkin, Yuval Blat, William J. Pitts, David S. Weinstein and James R. Burke

J. Biol. Chem. 2015, 290:11061-11074.
doi: 10.1074/jbc.M114.619502 originally published online March 11, 2015

Access the most updated version of this article at doi: 10.1074/jbc.M114.619502

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2015/03/11/M114.619502.DC1

This article cites 41 references, 14 of which can be accessed free at http://www.jbc.org/content/290/17/11061.full.html#ref-list-1