**ABSTRACT**

Background/Purpose: Targeting of the JAK-STAT pathway has been shown to be efficacious for treatment of patients with rheumatoid arthritis through the successful use of selective Janus kinase inhibitors in clinical trials. To date, several selective JAK3 inhibitors has faciliated the assessment of the role of JAK3 in autoimmune diseases. A JAK3 selective inhibitor has the potential benefit of avoiding undesirable side effects of JAK1 and JAK2 inhibition such as gastrointestinal intolerance, immunosuppression, and suppression of hematopoiesis. A new JAK3 selective inhibitor, Tofacitinib, was developed by Principia Biopharma. Tofacitinib is shown to be selective among the JAK family through a variety of assays.

Methods: Enzyme potencies were measured using the Caliper platform at NanoSyn Inc. (Santa Clara, CA). IL-2 stimulated phospho-STAT5 was measured in a NanoSyn’s Flex4 assay with a panel of human primary mononuclear cells (PBMCs) by flow cytometry. IL-4 stimulated STAT activation was determined using a NanoSyn’s Flex4 assay with a panel of human primary mononuclear cells (PBMCs) (horseigenes, Madison, WI). Protein profiling was performed at DiscoveRx (San Diego, CA).

Results: We have developed a series of molecules that are highly potent and selective for JAK3. Compound 1 inhibited JAK3 enzymatic activity with an IC50 of 0.5 ± 3.0 nM but not JAK1, JAK2, or JAK3 up to a concentration of 5 μM. The selectivity among other kinases within the Cys family was also high with no inhibition occurring beyond 1 μM. Protein profiling a panel of 442 kinases confirmed the exceptional selectivity of the series. Compound 1 forms a durable, reversible interaction with JAK3 in biochemical assays with a dissociation half-life of 8 hours. In cell-based assays, Compound 1 was highly potent and selective (IC50 7 nM and IC50 > 500 nM, respectively) in the IL-4-driven STAT activation model in Ramos B cells and in IL-2 driven STAT5 activation (IC50 > 240 ± 8 nM in PBMCs). IL-4 stimulated STAT activation was not inhibited up to 5 μM indicating complete cellular selectivity for JAK3 over JAK1. In addition, NFAT activation analysis through TCF driven luciferase reporter and 4T1 mammary tumor cells were not blocked.

Conclusions: Compound 1 is a potent, selective and durable inhibitor of JAK3 and has the potential to be an efficacious treatment for rheumatoid arthritis. In vitro cell based driven processes with a potential for differentiation from pan-JAK3 inhibitors.

**INTRODUCTION**

JAK3 Kinase 3 (JAK3) is a tyrosine kinase that links common gamma chain signaling to intracellular effector functions. This pathway regulates responses to IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Other family members include JAK1, JAK2 and TYK2 which control a wide variety of cellular functions.

JAK3 deficiency in humans is associated with a specific SCID phenotype that manifests as death due to T cells and NK cells. B cells are present but demonstrate impaired function. Since JAK3-SCID patients fail to induce immune cells and JAK3 selectin in mice also manifests as only a defective immune function, selective targeting of JAK3 may be a potential approach to treat immune- mediated disorders without avoiding various side effects.

Targeting of JAK3 selectively with small molecule inhibitors has been challenging due to the highly conserved amino acid composition of the ATP binding site, i.e., the JAK family members share the same three amino acids in the binding pocket, Cys, to be used to count the selectivity among the JAK family. The unique JAK3 residue, Cys, is involved in a short motif of a covalent reaction. This provides a mechanism to achieve selectivity versus JAK1, JAK2 and JAK3. Small molecules chemistry that allows the covalent Cys interaction to be reversible, Principia has produced selective JAK3 inhibitors that will not form protein adducts which has been a concerning factor of previous Cys targeting chronic drugs.

The combination of covalent modification of Cys along with targeting a unique gatekeeper amino acid has allowed the identification of a highly selective, small molecule JAK3 inhibitor.

**RESULTS**

**Compound 1: Biochemical screening shows selectivity for JAK3**

**Alignment of JAK3 Cys family kinases**

<table>
<thead>
<tr>
<th>Kinase</th>
<th>IC50 (uM)</th>
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<tbody>
<tr>
<td>JAK3</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>JAK1</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>JAK2</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>TYK2</td>
<td>&gt; 500</td>
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</table>

**High selectivity for JAK3 among other JAK family members.**

Tofacitinib inhibited JAK3 with an IC50 of 0.3 ± 0.2 uM while JAK1, JAK2, and TYK2 were not inhibited. In the IL-4 pStat6 assay, Compound 1 inhibited JAK3 with an IC50 of 0.64 ± 0.08 uM whereas JAK1, JAK2, and TYK2 were not inhibited. In the IL-2 induced pSTAT3 assay, JAK3 was inhibited with an IC50 of 0.03 ± 0.00 uM while JAK1, JAK2, and TYK2 were not inhibited. These results show that Compound 1 is selectively inhibits JAK3 for both JAK-dependent and independent pathways.

**Reversible covalent JAK3 inhibitor demonstrates slow off-rate kinetics**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Off-rate constant (min-1)</th>
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<tr>
<td>JAK3</td>
<td>2.3</td>
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</table>

JAK3 inhibition occurs by a fluorescence resonance energy transfer (FRET) probe associated with IL-2 induced T cells. The KIPYIV peptide was not cleaved by human JAK3, leading to the formation of the JAK3-LSDYLRTQRGL-APICLV fragment. To demonstrate the reversibility of the JAK3 inhibitor, Cell viability after 72hrs. was measured with CellTiter-Glo. JAK3 inhibition leads to a decrease in cell viability. RCAM treatment with the JAK3 inhibitor leads to a decrease in cell viability.

**DISCUSSIONS**

Cytokine targeting by covalent binding allows complete JAK3 selectivity within the JAK family of kinases as well as excellent on-target selectivity. Use of reversible covalent chemistry produces inhibitors with slow off-rate kinetics allowing durable inhibition of the target.13 Principia has identified a highly potent and selective inhibitor of JAK3 that can be utilized to probe the effects of selective JAK3 inhibition. Selective JAK3 inhibition is sufficient to completely block JAK-STAT signaling and function in primary human T cells as well as IL-4 induced signaling in Ramos B cells. Selective, potent, JAK3 inhibitors have the potential to be differentiated from pan-JAK inhibitors on the basis of JAK2-driven side effects.

**References**


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**Discovery of a highly potent, selective, reversible covalent inhibitor of JAK3**

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