TITLE: Off-target profiling of tofacitinib and baricitinib by machine learning: a focus on thrombosis and viral infection

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AMB: Study conception, data interpretation and critical revisions of the manuscript.
GS: Analysis of results, data interpretation, revisions to the manuscript.
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ABSTRACT:

Objectives: There are no clear on-target mechanisms that explain the increased risk for thrombosis and viral infection or reactivation associated with Janus kinase (JAK) inhibitors. We aimed to identify and validate off-target binding effects of the JAK inhibitors baricitinib and tofacitinib using computational and experimental methods.

Methods: Potential biological targets of baricitinib and tofacitinib were predicted using two established computational methods. Targets related to thrombosis or viral infection/reactivation were experimentally validated using biochemical and cell-based in vitro assays.

Results: Overall, 98 targets were predicted by the computational methods (baricitinib n=41; tofacitinib n=58), of which 17 drug-target pairs were related to thrombosis (n=10) or viral infection/reactivation (n=7), and 11 were commercially available for in vitro analysis. Inhibitory activity was identified in vitro for four drug-target pairs – two related to thrombosis in the micromolar range (phosphodiesterase 10A [baricitinib], transient receptor potential cation channel subfamily M subtype 6 [tofacitinib]) and two related to viral infection/reactivation in the nanomolar range (Serine/threonine protein kinase N2 [baricitinib, tofacitinib]).

Conclusions: Previously unknown off-target interactions for the two JAK inhibitors were identified. The proposed pharmacological off-target effects include attenuation of pulmonary vascular remodeling, modulation of Hepatitis C viral response and hypomagnesemia. Off-target effects related to an increased risk of thrombosis or viral infection/reactivation for baricitinib and tofacitinib were not identified. Further clinical and experimental research is required to explain the observed thrombosis and viral infection/reactivation risk.

KEYWORDS: JAK inhibitor; off-target effect; drug safety; drug repurposing; thrombosis.
INTRODUCTION:

Janus Kinase (JAK) inhibitors are novel targeted synthetic disease-modifying antirheumatic drugs (tsDMARDs). The new class of small molecule drugs represents an important alternative to treat moderate-to-severe rheumatoid arthritis (RA) patients with non- or inadequate-response to conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) and biological disease-modifying antirheumatic drugs (bDMARDs).(1) The JAK inhibitors target one or more kinases of the JAK family (JAK1, JAK2, JAK3, and non-receptor tyrosine-protein kinase TYK2) and inhibit multiple pro-inflammatory cytokines, such as interleukin (IL)-6, IL-10, and interferon (IFN)-γ.(2,3) Baricitinib (JAK1/JAK2 inhibitor) and tofacitinib (JAK1/JAK3 inhibitor) are the first members of this class approved in the United States (US) and Europe to treat RA (Figure 1).

![Chemical structure of baricitinib and tofacitinib](image)

**Figure 1.** Chemical structure of baricitinib and tofacitinib, the first JAK inhibitors approved in the USA and Europe to treat rheumatoid arthritis.

Safety concerns associated with JAK inhibitors, such as the increased risk for thrombosis and viral infection or reactivation have emerged worldwide and boxed warnings are included on all approved JAK inhibitors used to treat inflammatory conditions.(4–7) While a dose-response effect was observed in the risk of thrombosis in clinical trials of both baricitinib and tofacitinib, there is no clear mechanism associated to the
pharmacological target that could explain the risk of thrombosis associated with JAK inhibitors. Thus, the increased risk of these safety concerns is heavily debated.

It is well established that unintended off-target activity may interfere in multiple biological processes, inducing unwanted side effects.(8) In this context, computational approaches, such as machine learning, can be used to predict the potential for an approved drug to interact with off-targets and identify potential safety-related concerns.(9,10) For example, previously unknown drug-target interactions for the approved compound Celecoxib were identified, supporting the biological plausibility of reported cardiovascular adverse drug events.(11) Off-target profiling is frequently used to identify candidate drugs for repurposing. For example, computational studies identified baricitinib as a promising JAK inhibitor for repurposing in patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2 or COVID-19).(12,13) Baricitinib was considered to be a therapeutic option based on the high affinity for AP-2 associated protein kinase 1 (AAK1), which is key in regulating viral endocytosis(12) and its inhibition may reduce the ability of the virus to infect lung cells.(14)

In light of the currently unexplained thrombotic and viral infection risk, and the previously observed off-target binding potential of baricitinib, we sought to identify if the thrombosis and viral infection/reactivation risk may be a result of an off-target effect. We therefore aimed to leverage computational methods to identify unexplored drug-protein interactions for baricitinib and tofacitinib in vitro.

**METHODS:**

**Macromolecular target prediction and selection.**

Macromolecular targets of baricitinib and tofacitinib were predicted using two machine learning approaches, Target Inference Generator (TIGER v. 19.07)(11) and SOM-based Prediction of Drug Equivalence Relationships (SPiDER)(15). TIGER and SPiDER leverage self-organizing maps(16) for target prediction (see online supplementary material 1). Targets with statistically meaningful predictions from SPiDER (p<0.05) and/or TIGER (score>1) were selected for in vitro testing if they were considered to have a potential influence in thrombosis and viral infection/reactivation.
In vitro characterization.

Baricitinib (99.97% purity) and tofacitinib (99.96% purity) compounds were purchased from MedChem Express LLC (New Jersey, www.medchemexpress.com). In vitro characterization was performed on a fee-for-service basis at Eurofins (www.eurofins.com), if the assay was commercially available. Baricitinib and tofacitinib were tested at a single concentration (30 µM) or multiple concentrations (30 µM highest concentration) with technical replicates, using biochemical assays (radioligand or enzymatic assays) or cell-based assays (see online supplementary material 2 for further details).

For cell-based assays, dose-response curves and corresponding the half-maximal inhibitory/effective concentrations (IC$_{50}$/EC$_{50}$) were determined. For the biochemical assays, compound-targets showing an experimental readout greater than 25% (inhibition or stimulation) at 30 µM were selected for follow-up, and dose-response curve characterization and determination IC$_{50}$/EC$_{50}$ (two or three replicates, multiple concentrations, maximum 100 µM concentration).

IC$_{50}$ values were determined by non-linear least squares regression analysis, using RStudio v. 1.3.1073 (PBC, Boston, MA, http://www.rstudio.com/), MathIQTM (ID Business Solutions Ltd., UK) and GraphPad Prisma v. 9.1.0 (GraphPad Software, San Diego, CA). Inhibitory constant (K) values were estimated from experimental IC$_{50}$ values using a web based tool(17) based on the equation of Cheng and Prusoff(18) and the observed IC$_{50}$ of the tested compound.

Docking analysis.

Computational ligand docking was use to gather potential mechanistic insights into experimentally observed drug-target interactions. We used GOLD (Cambridge Crystallographic Data Centre, https://www.ccdc.cam.ac.uk/) within Molecular Operating Environment (MOE) software (v.2019.0102; Chemical Computing Group ULC, Montreal, QC)(19). The scoring function (GoldScore scoring [PDB ID: 4CRS] and PLP scoring [PDB ID: 5C1W]) was chosen to minimize the RMSD in re-docking, always obtaining a RMSD lower or equal to 0.80 Å. For each drug-target pair, the best-scoring docking pose was analyzed (online supplementary material 3).
RESULTS:

The target prediction methods identified 40 potential targets for baricitinib and 58 for tofacitinib (online supplementary tables 1 and 2, respectively). From all predicted targets, five targets for baricitinib and five for tofacitinib were identified as being relevant for thrombosis (Table 1). For viral infection/reactivation (Table 2), four targets were identified for baricitinib and three for tofacitinib.

Table 1. Suggested targets with impact on thrombosis stratified by JAK inhibitor.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Protein Kinase C Beta (PKC-β)</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Adenosine Receptor A2A (AA2AR)</strong></td>
</tr>
<tr>
<td><strong>Baricitinib</strong></td>
<td><strong>Inducible Nitric Oxide Synthase (iNOS)</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Phosphodiesterase 10A (PDE10A)</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Ras Related Protein Rab-7a</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Arachidonate 15-Lipoxygenase (15-ALOX)</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Adenosine Receptor A2A (AA2AR)</strong></td>
</tr>
<tr>
<td><strong>Tofacitinib</strong></td>
<td><strong>Short transient receptor potential channel 6 (TRPC6)</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Short transient receptor potential channel 3 (TRPC3)</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Adenosine Receptor A3 (ADORA3)</strong></td>
</tr>
</tbody>
</table>

*Commercial assays were unavailable for TRPC6 or TRPC3, and therefore, these targets could not be validated. Instead, transient receptor potential cation channel subfamily M member 6 (TRPM6) was employed for the respective binding assays.
Table 2. Suggested targets with impact on viral infection or reactivation stratified by JAK inhibitor.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baricitinib</td>
<td>Epidermal growth factor receptor (EGFR) kinase</td>
</tr>
<tr>
<td></td>
<td>Deoxycytidine kinase (DCK)</td>
</tr>
<tr>
<td></td>
<td>Serine/threonine-protein kinase N2 (PKN2)*</td>
</tr>
<tr>
<td></td>
<td>Thymidine kinase (HSV)</td>
</tr>
<tr>
<td>Tofacitinib</td>
<td>Exportin-1 (XPO1)</td>
</tr>
<tr>
<td></td>
<td>Serine/threonine-protein kinase N2 (PKN2)</td>
</tr>
<tr>
<td></td>
<td>Ubiquitin-conjugating enzyme E2 N (Ubc13)</td>
</tr>
</tbody>
</table>

* PKN2 was included in the list of targets tested for baricitinib, which allowed us to make a direct comparison between tofacitinib and baricitinib inhibitory activity on this target.

Of the 98 predicted targets, a total of 11 drug-target interactions were experimentally validated based on the availability of fee-based in vitro testing services (Table 3). Among predicted targets, two members of the Transient Receptor Potential superfamily of calcium channels were suggested, namely short transient receptor potential channels 6 (TRPC6) and 3 (TRPC3). Commercial assays were unavailable for TRPC6 or TRPC3, and therefore, these targets could not be validated. Instead, transient receptor potential cation channel subfamily M member 6 (TRPM6) was employed for the respective binding assays.

Additionally, while serine/threonine-protein kinase N2 (PKN2) was among the predicted targets for tofacitinib, but not for baricitinib, PKN2-baricitinib binding affinity was previously determined in baricitinib (apparent dissociation constant \( K_{d}^{\text{app}} \) = 269 nM and \( IC_{50} = 284 \text{ nM} \). Thus, PKN2 was included in the list of targets tested for baricitinib, allowing a direct comparison between tofacitinib and baricitinib inhibitory activity on this target.

From the 11 drug-target interactions tested, five showed an experimental readout greater than 25% drug-target interaction at 30 \( \mu \text{M} \), and were selected for further in vitro characterization (Table 3). Four out of five drug-target interactions were confirmed by further in vitro evaluation. \( IC_{50} \) and \( K_{i} \) or \( K_{d} \) values in the nanomolar range (baricitinib and tofacitinib on PKN2) and in the micromolar range (baricitinib on Phosphodiesterase 10A2
PDE10A2; tofacitinib on TRPM6) were determined. Targets for which the IC$_{50}$ was greater than 30 µM were defined as “inactive” (Table 3). The raw in vitro data for drug-binding activity using biochemical assays is available in the online supplementary table 3. Dose-response curves for targets showing activity are available as Supplementary Material 4.

Table 3. In vitro findings for baricitinib and tofacitinib off-target activity. JAK inhibitors were tested at a concentration of 30 µM.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Safety issue</th>
<th>Predicted target</th>
<th>IC$_{50}$ (µM)</th>
<th>$K_i$ or $K_d$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baricitinib</td>
<td>Thrombosis</td>
<td>Adenosine Receptor A2A (AA2AR)$^{[a]}$</td>
<td>Inactive</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inducible NOS (iNOS)</td>
<td>Inactive</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PI3 Kinase (p110b/p85a)</td>
<td>Inactive</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phosphodiesterase 10A2 (PDE10A2)$^{[b]}$</td>
<td>28 ± 2 $^{[c][d]}$</td>
<td>$K_i = 6.1$</td>
</tr>
<tr>
<td></td>
<td>Viral infection</td>
<td>Serine/threonine-protein kinase N2 (PKN2)</td>
<td>0.24, 0.21 $^{[d][e]}$</td>
<td>$K_i = 0.082, 0.069$ $^{[e]}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Epidermal growth factor receptor (EGFR)</td>
<td>Inactive</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tofacitinib</td>
<td>Thrombosis</td>
<td>Adenosine Receptor A3 (ADOR3)$^{[a,b]}$</td>
<td>Inactive</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transient receptor potential cation channel subfamily M member 6 (TRPM6)*</td>
<td>n.d.</td>
<td>$K_d = 6.1, 7.7^{[e]}$</td>
</tr>
<tr>
<td></td>
<td>Viral infection</td>
<td>Adenosine Receptor A2A (AA2AR)$^{[b]}$</td>
<td>Inactive</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Serine/threonine-protein kinase N2 (PKN2)</td>
<td>0.71, 0.74 $^{[d][e]}$</td>
<td>$K_i = 0.24, 0.25$ $^{[e]}$</td>
</tr>
</tbody>
</table>

[a] Antagonistic effect
[b] Agonistic effect
[c] values are the mean ± standard error of the mean (SEM) for n > 2.
[d] During follow-up experiments, JAK inhibitors were tested in multiple concentrations (top concentration of 100 µM) for dose-response curve characterization and determination IC50/EC50 (two or three replicates).
[e] For number of replicates (n) > 2, mean and standard error of the mean (SEM) are presented. For n =2, no averaging was made and both values are presented.
*n.d.: not determined

Commercial assays were unavailable for TRPC6 or TRPC3, and therefore, these targets could not be validated. Instead, transient receptor potential cation channel subfamily M member 6 (TRPM6) was employed for the respective binding assays.

Note: All in vitro testing was performed on a fee-for-service basis at Eurofins Cerep (www.eurofins.com)
Computational ligand docking (Figure 2) predicts potential modes of interaction for baricitinib and tofacitinib in the binding pocket of the identified macromolecular targets (PKN2 [PDB-ID: 4CRS(21)]; PDE10A [PDB-ID: 5C1W(22)]). TRPM6 was not considered due to the unavailability of an experimentally determined structure. Further information on the computational docking is available as Supplementary Material 5.

Figure 2. Predicted docking pose of baricitinib (magenta, A) and tofacitinib (cyan, B) in the binding site of PKN2 (PDB-ID: 4CRS). Predicted binding pose of baricitinib (magenta, C) in the binding site of PDE10A (PDB-ID: 5C1W).

**DISCUSSION:**

The confirmed drug-target interactions suggest an attenuation of pulmonary vascular remodeling (inhibition of PDE10A), modulation of Hepatitis C (HCV) viral response (inhibition of PKN2), and hypomagnesemia (inhibition of TRPM6). Therefore, we did not
identify off-target effects that could explain the elevated thrombosis or viral infections/reactivation risk observed in the clinical setting.(4,23,24) Both baricitinib and tofacitinib were confirmed as “promiscuous drugs”, since they bind to proteins from families other than the primary therapeutic target.(11) Thus, both drugs may be potential candidates for adverse drug effects and further repurposing.

The thrombotic and cardiovascular risk associated with JAK inhibitors remains debated, which is largely due to a lack of a clear mechanism associated to the therapeutic target that could explain the increased risk. In our analysis, we aimed to investigate if there may be a plausible off-target drug-effect. While the computational approaches identified several targets relevant for blood coagulation and platelet aggregation (e.g., Adenosine receptor A2A [AA2AR] and Arachidonate 15-lipoxygenase [15-ALOX]), neither baricitinib nor tofacitinib were found to interact with those receptors in vitro, ruling them out as potential off-targets.

Nonetheless, the drugs were shown to inhibit two targets related to thrombosis – PDE10A and TRPM6. PDE10A, which was recently validated as a novel target to treat pulmonary arterial hypertension (PAH) due to its central role in progressive pulmonary vascular remodeling,(25,26) was identified as a target of baricitinib. The preliminary in vitro results of this study showed moderate inhibition of baricitinib for PDE10A2. Molecular docking in the active site of PDE10A showed in Figure 2 suggested a similar binding pose of baricitinib to the crystallized inhibitor (PDB ID: 5C1W), with a predicted \( \pi \)-stacking interaction with the Phe719 residue, crucial for biological activity.(27) Additionally, among important regions for ligand binding is the occupation of a hydrophobic clamp formed by two phenylalanine residues, Phe719 and Phe686.(27,28) Clinically, PDE10A inhibition is expected to decrease the risk for thrombosis, particularly in patients with PAH. Thus, the expected positive clinical impact of PDE10A inhibition on the risk of thrombosis is not in line with a potential link to an elevated thrombosis risk. Rather, baricitinib might improve progressive pulmonary vascular remodeling.

This study further identified previously unknown off-target interactions of tofacitinib on the TRPM6, with moderate binding affinity. While our computational approach identified TRPC6 and TRPC3 as potential targets, we were unable to experimentally validate these
targets due to lack of commercially-available in vitro assays. Thus, we can only speculate that the binding affinity observed with TRPM6 may translate to binding in TRPC6 and TRPC3. Additional experiments are needed to confirm if the C subfamily is also a potential target of tofacitinib. This is important as TRPC6 is known to regulate human clot retraction, physiological hemostasis, and thrombus formation and its inhibition is thought to have a positive effect on thrombotic outcomes.(29) Thus, further research is needed to confirm if there is an off-target activity of tofacitinib-TRPC6.

Cumulatively, the active targets in this study suggest that JAK inhibitors may have a beneficial effect on cardiovascular risk, and therefore do not support a hypothesis that the risk of thrombosis is related to an off-target drug effect (in the framework of the macromolecular targets investigated in this study). Nevertheless, we note that recent US-based cohort studies that have identified no difference in thrombosis risk between tofacitinib and TNF-inhibitors,(30–32) thereby suggesting much of the observed risk seen in pharmacovigilance studies(6,5) may be due to underlying risk factors rather than a drug effect. For example, standardized incidence rates (IR) of venous thromboembolism (VTE) or pulmonary embolism (PE) were comparable among patients with RA using tofacitinib (IR = 1.05 [0.78-1.39]) and bDMARDs (IR = 0.94 [0.85-1.03]) within MarketScan database cohorts.(32) Conversely, a recent analysis using pharmacovigilance data of the US FDA Adverse Event Report System (FAERS) did not identify a signal of disproportionate reporting for VTE and/or PE events with tofacitinib. Therefore, improved understanding of the underlying risk-factors for thrombosis in patients with JAK inhibitors is urgently needed.

The risk of thrombosis can be further increased in RA patients with high disease activity, cardiovascular risk factors (e.g., obesity), immobility, and hormonal replacement therapy.(33,34) Patients using JAK inhibitors frequently have high disease activity with non- or inadequate-response to csDMARDs and bDMARDs and multiple chronic conditions (e.g., cardiovascular disorders and depression), which can make the attribution of thrombotic events in patients treated with JAK inhibitors even more intricate.

In addition to thrombosis, targets related to viral infection and viral reactivation were investigated. Therapies targeting the JAK family of enzymes may interfere with normal
antiviral response including inhibition of IFN-γ activity and may potentially increase the risk of infection/ reactivation of several viral infectious diseases, particularly HZ, in RA patients.(23,35) The computational approaches identified several targets expected to play a role in viral endocytosis and viral response, including epidermal growth factor receptor (EGFR) kinase and PKN2. Although, baricitinib and tofacitinib were not found to interact with EGFR in the assays used, PKN2 inhibitory activity was identified for baricitinib and tofacitinib.

Clinically, PRK2 is of great importance as a target for antiviral therapy, particularly anti-HCV, as its suppression leads to viral replication blockage in humans.(36) PKN2 inhibitors in combination with other antiviral therapies have demonstrated synergistic antiviral activity for chronic HCV treatment.(37,38) To date three studies have evaluated tofacitinib binding activity on PKN2, however results are contradictory.(20,39,40) The preliminary in vitro results of the current study suggest PKN2 inhibition with both baricitinib and tofacitinib, as the IC_{50} and K_i values are in the nanomolar range. Moreover, the molecular docking in the PKN2 crystalized structure (PDB-ID: 4CRS) suggested a similar binding mode, shape, and certain molecular features (i.e. pharmacophore) of baricitinib and tofacitinib as to the co-crystalized ligand at the protein binding site. The model indicates that the two drugs interact with the Mg^{2+} similarly to the crystallized ligand – a PKN2 inhibitor – on the kinase functional pocket.(41) However, the impact of PKN2 inhibition is proposed to have a positive effect on viral suppression, and therefore does not explain the elevated risk of HZ in RA patients. The exact mechanism of HZ viral reactivation remains unclear.

Outside of its role in viral suppression, PKN2 may play an essential role in various cellular processes, such as cellular proliferation, migration, and signaling pathways.(42–44) Moreover, PKN2 is involved in autoinflammatory disorders,(45) heart failure,(46) and it is a target of interest in cancer.(44,47,48) As concerns regarding the risk of malignancy and major adverse cardiovascular events (MACE) in patients treated with tofacitinib have been raised by the European Medicines Agency (EMA), it is important to consider the potential role of PKN2 inhibition.(49) However, in mice models, PKN2 activation was the cause of cardiac dysfunctions,(46) and therefore, the clinical impact of PKN2 inhibition is contradictory to the risk of cancer and MACE in RA patients.
Off-target profiling using computational approaches has been widely used to identify candidates for drug repurposing.(50,51) Indeed, JAK inhibitors were recently established as potential candidate therapies for SARS-CoV-2 based on in silico methods.(52–54) Our computational methods identified 98 drug-target predictions, and the preliminary in vitro results found inhibitory activity on several proteins other than the primary therapeutic target, thereby confirming baricitinib and tofacitinib as promiscuous drugs(55,56) and candidates for drug repurposing studies. For example, PDE10A inhibition has been primarily studied in psychiatric and neurological conditions, such as schizophrenia(57) and Huntington’s disease,(58) and, to a lesser extent, in multiple peripheral pathological conditions(59,60) (e.g., osteogenic differentiation). Additionally, PDE10A inhibition by baricitinib is hypothesized to have a synergistic pharmacological effect in combination with other COVID-19 treatments (e.g., antiviral and corticosteroids drugs), due to the anti-fibrotic and anti-inflammatory effects of phosphodiesterase’s inhibitors on the treatment of COVID-19 and its associated conditions (e.g., thrombosis, inflammation, and fibrosis).(61,62) Therefore, the confirmed PDE10A inhibition identified in this study supports the potential for baricitinib as a potential candidate outside of rheumatology. Moreover, while TRPM6 was not initially predicted the moderate inhibitory active is worth investigating. TRPM6 inhibition is not fully elucidated, however, it is mainly involved in magnesium homeostasis in the intestine and kidney(63,64) and it has been demonstrated to have expression levels modulated by hormones such as estrogen(65) and angiotensin II,(66) immunosuppressant(67) and diuretics drugs,(68) and epidermal growth factor (EGF).(69) Moreover, the decreased expression of TRPM6 in cancer patients treated with EGFR targeted therapies (e.g., cetuximab) seems to positively contribute to the oncologic efficacy of these therapies, as decreased magnesium availability inhibits cell proliferation and slows down tumor growth.(70,71) Thus, we encourage further investigation on clinical relevance of TRPM6 inhibition by tofacitinib in oncology.

Only a small fraction (~10%) of the 98 predicted off-targets were experimental tested in this study. However, as we did observe active binding on 3 distinct targets, this study suggests that there might be other interactions among the list of predicted targets. Thus, further testing might help to elucidate the molecular mechanisms of JAK inhibitors, and
open the door for improved understanding of the safety concerns and repurposing in other conditions (e.g., in neurodegenerative diseases, diabetes and viral infections).

The use of computational and experimental approaches in this study allowed for the identification and characterization of previously unknown off-target interactions for the two JAK inhibitors (e.g., baricitinib-PDE10A and tofacitinib-TRPM6), which adds to the known literature on the potential drug-effects of these drugs. Moreover, the reference annotated targets used by the computational methods(72) encompasses a broad scope of protein families, which may allow identification of drug promiscuity and predict a broad off-target drug profile, pointing out potential targets for repurposing studies.

Nevertheless, despite the strengths of the study we are mindful of some limitations. As identified, we could not experimentally validate all predicted targets related to thrombosis (e.g., TRPC6) or viral infection/reactivation (e.g., deoxycytidine kinase [DCK], Thymidine kinase [HSV], Exportin-1 [XPO1], or Ubiquitin-conjugating enzyme E2 N [Ube13]). As such, we cannot conclude if these targets may play an important role in thrombosis or viral infection/reactivation risk and are limited in the conclusions we can draw. Thus, we encourage researchers with access to the appropriate assays to validate these targets. Moreover, there might be additional targets of relevance that were not predicted by our computational tools. We also acknowledge that the activity of small molecule drugs using in vitro assays does not always translate into activity in the cellular environment. Thus, the results should still be interpreted with caution and treated as preliminary evidence for the off-target binding of baricitinib and tofacitinib.

**CONCLUSION:** The combination of computational methods and experimental validation identified and characterized previously unknown off-targets of baricitinib and tofacitinib. The confirmed target interactions suggest an attenuation of pulmonary vascular remodeling, modulation of HCV viral response, and hypomagnesemia, thus it does not endorse the hypothesis of elevated thrombosis or viral infections/reactivation risk explained by one (or more) drug-target interactions. Consequently, the current safety concerns may be due to underlying patient-specific factors (confounders) or to targets not detected by our computational pipeline, but as not all of the predicted targets related to thrombosis or viral infection/reactivation were experimentally validated further
research is warranted. Finally, baricitinib and tofacitinib may be potential candidates for repurposing, as they were identified as drugs with promiscuous binding activity.

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