Discovery of novel Janus kinase inhibitors by virtual screening

Thesis summary

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1. **INTRODUCTION AND BACKGROUND**

The primary motivation of drug discovery is improving life expectations and the quality of life by combating diseases. While it is a long and expensive process (the average cost of introducing a new drug is estimated to be more than 2.5 billion USD in the most recent study of DiMasi et al.\(^1\)), computer-aided drug design can be a viable strategy to increase the effectiveness, to shorten and rationalize costs in early research phases.

Abnormalities in inter- and intracellular signaling are of great importance in the pathology of diseases with high unmet medical need (e.g. cancer and inflammatory diseases). Small-molecule modulation of certain signaling pathways (for example with the inhibition of some enzymes in the pathway) has been proposed and proven as an opportunity for pharmaceutical intervention.

Protein kinases are key elements of signaling pathways and constitute a family with more than 600 enzymes. Their function is to catalyze phosphorylation (the transfer of a phosphoryl group from ATP to a protein), which is the driving process of many cellular signaling pathways (or signaling cascades), hence kinases have diverse and important roles in the living organism. The role of protein kinases has been (and continues to be) established in a vast number of diseases, including oncological, autoimmune and inflammatory diseases, thus they are one of the two large protein families that are most targeted in drug discovery (the other being G-protein coupled receptors).\(^2\) Small-molecule inhibition of kinases has been proposed and applied for the

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pharmaceutical intervention of various oncological and inflammatory diseases.³

Janus kinases (JAKs) are a family of kinases associated with cytokine receptors. The family consists of four enzymes: JAK1, JAK2, JAK3 and TYK2. Their main function is the regulation of the JAK/STAT intracellular signaling pathways, which affect gene transcription.⁴ A quick summary of the main steps of JAK/STAT signaling is presented in Figure 1. The JAK/STAT pathways are involved in a number of physiological processes, primarily inflammation and immune responses. Malfunctions of these pathways are linked to the development of a number of oncological and inflammatory diseases, such as polycythemia vera or rheumatoid arthritis.

Currently, there are two marketed JAK inhibitors: the JAK1/JAK2 inhibitor ruxolitinib, approved in late 2011 for the treatment of myelofibrosis, and the pan-JAK inhibitor tofacitinib, approved a year later for the treatment of rheumatoid arthritis. While both have met the strict safety and effectivity criteria that are required for FDA approval, these drugs exhibit only limited subtype selectivities within the JAK family, which ultimately contributes to their reported side effects. (As a result, tofacitinib was not approved for distribution in the European Union in 2015.) Furthermore, they are not specific to somatic JAK mutants, such as JAK1V658F and JAK2V617F that are connected to acute lymphoblastic leukemia and polycythemia vera, respectively. Consequently, selective JAK inhibitors are still very much sought after.

The main steps of JAK/STAT signaling: binding of the extracellular signaling molecule (cytokine) to the respective receptor is followed by JAK activation, which facilitates the dimerization of the STAT (Signal Transducer and Activator of Transcription) proteins via phosphorylation. The STAT dimer then enters the nucleus and activates DNA transcription. (Source: https://courses.washington.edu/conj/bess/jakstat/jakstat.htm)

However, the design of subtype selective compounds is a tough challenge due to the high sequential homology and structural similarity of the ATP binding sites of the JAK isoforms, especially in the JAK2 vs. JAK1 relation.

In my thesis, I have applied computational drug discovery methods to identify novel inhibitors of Janus kinases, particularly JAK1 and JAK2. Besides applying many computational methods, I have also developed specialized solutions to some of the subtasks I have
encountered along the way. This includes the development of a pre-screening filter for kinase-directed virtual screening (Kinase Desirability Score or KiDS) and a scoring method based on interaction fingerprints (IFP) for the prediction of JAK2 selectivity. During the virtual screening campaigns presented in this thesis, we have identified several new inhibitors of JAK1, JAK1\textsuperscript{V658F} and JAK2.

2. METHODS

Virtual screening has been a well-established collection of methods for providing hit compounds for drug discovery for almost twenty years now.\textsuperscript{5} It encompasses structure-based methods that account for structural information about the target protein or its complex with a ligand, and ligand-based methods that account only for the chemical structures and properties of the already known and prospective ligands and their similarities. From structure-based methods, ligand docking is the most popular one: it involves the generation of 3D ligand conformers inside a binding pocket and scoring the resulting binding conformations (binding poses) with a suitable scoring function to give a (rough) estimate of their binding affinity.

Molecular dynamics (MD) is a simulation method for studying the dynamic behavior of molecular systems. During an MD simulation, potential energies of the atoms, as well as forces between them, are evaluated in small time intervals (time steps). The output trajectory of the system contains the motions of the atoms over time. During this work, I have used MD simulations to provide protein conformers for ligand docking.

Molecular fingerprints provide numerical descriptions of the

structure or certain features of molecules, thus enabling the quantification of the similarity of two molecules with a suitable similarity metric, such as the Tanimoto coefficient. In particular, interaction fingerprints (IFP) encode information about the interactions between the molecule and its environment – usually a protein target –, enabling the comparison of experimentally resolved or computationally docked protein-ligand complexes.\(^6\)

Multi-parameter optimization (MPO) is a collective name for methods that aid the simultaneous optimization of several properties. For compound profile optimization, a suitable MPO method is the desirability function that assigns a “desirability” score for each possible value of each property. I have utilized desirability functions during the development of the Kinase Desirability Score (KiDS).

For the retrospective evaluation of KiDS, and the developed virtual screening workflows, we have applied standard evaluation metrics, such as enrichment factors (the likeliness to find actives in the top x% of the scored dataset, compared to selecting randomly) and area under the ROC curve values (the probability that a randomly selected active molecule receives a better score than a random inactive).

### 3. RESULTS

3.1 Property-based scoring scheme for kinase-like ligands

This part of the work was prompted by a need to rationalize the computational resources allocated to the ligand docking step of some of the virtual screenings presented in the following subsections. For this purpose, a pre-screening filter for „kinase-like” ligands (compounds

with a higher likeliness of kinase activity) can be applied. Various approaches have been developed previously for this purpose, including substructure- and similarity-based methods, and a property-based scoring scheme called Kinase-Like Score (KLS).\(^7\)

In this work, we have applied desirability functions to develop a scoring scheme for the efficient filtering of kinase-like ligands. Several commonly used molecular descriptors were examined to select a combination of them with an optimized performance in discriminating known kinase ligands (obtained from the ChEMBL database) from random subsets of commercially available compounds (from the Mcule database). Finally, six descriptors were chosen to be included in the Kinase Desirability Score: topological polar surface area (TPSA), the number of rotatable bonds (rotB), nitrogen atoms (N\(_N\)), oxygen atoms (N\(_O\)), aromatic rings (Arom) and hydrogen bond donors (HBD). Each property can contribute with a desirability value between 0 and 1, thus the available values for the KiDS score are between 0 and 6.

The resulting scoring scheme was evaluated on a number of independent datasets – including publicly available and proprietary high-throughput screening (HTS) data – with standard evaluation metrics, such as enrichment factors and area under the ROC curve (AUC) values that are routinely used in the development of virtual screening protocols. It was concluded that KiDS have outperformed the Kinase-Like Score in terms of the evaluated performance metrics (see Table 1) and that it is also effective in selecting a subset enriched in kinase ligands from HTS datasets.

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Table 1. Performance evaluation of the Kinase Desirability Score: early enrichment factors and AUC values

<table>
<thead>
<tr>
<th>Dataset</th>
<th>EF$_{0.5%}$</th>
<th>EF$_{1%}$</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KiDS</td>
<td>KLS$^a$</td>
<td>KiDS</td>
</tr>
<tr>
<td>Training</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>23.2 (0.019)$^b$</td>
<td>1.90 (5.1E-3)</td>
<td>14.2 (0.01)</td>
</tr>
<tr>
<td>Test 1</td>
<td>22.6 (0.024)</td>
<td>1.87 (6.5E-3)</td>
<td>14.0 (0.013)</td>
</tr>
<tr>
<td>Test 2</td>
<td>18.9 (0.061)</td>
<td>3.78 (0.026)</td>
<td>14.8 (0.036)</td>
</tr>
</tbody>
</table>

$^a$ Performance parameters obtained for the same datasets with the KLS score of Singh et al. are provided for comparison.

$^b$ 95% confidence intervals are given in parentheses.

Based on the good and consistence performance of the Kinase Desirability Score, we have applied it as a pre-screening step (prior to ligand docking) in two of the virtual screening studies presented in this thesis.

3.2 Virtual screening for inhibitors of JAK1$^{V658F}$

As part of a collaboration with the research group of Prof. Peter P. Sayeski at the University of Florida, we have carried out a virtual screening against the NCI (National Cancer Institute) database to identify novel inhibitors of JAK1 carrying the V658F mutation, associated with acute lymphoblastic leukemia.

At the time of this study, there were two X-ray structures available for the human JAK1 enzyme. To develop an efficient virtual screening protocol, we have conducted a retrospective screening against both X-ray structures on a dataset of 18 known JAK1 inhibitors and 1782
decoy molecules, using the online virtual screening tools of Mcule.\(^8\) This involved the selection of the best X-ray structure and the optimization of two hydrogen bond constraints between the ligands and the hinge region of JAK1 (a loop connecting two larger domains in kinases, which is important for the binding of ATP and inhibitors).

In a prospective screening, 150,663 molecules from the NCI database were docked into the binding site of JAK1 (PDB: 3EYG) with the developed protocol. Of these molecules, 8136 were found with at least one binding pose satisfying the hydrogen bond constraints. The highest ranked 500 compounds from these were subjected to diversity selection, and the 250 most diverse molecules were selected as virtual hits. Of the requested compounds, 80 have arrived to our laboratories from NCI and after a DMSO solubility test, 71 were investigated experimentally in the laboratory of Prof. Sayeski for cell growth inhibition against JAK1 dependent cells (BaF3/JAK1\(^{V658F}\) cell line) carrying the V658F somatic mutation found in both acute lymphoblastic leukemia and acute myeloid leukemia patients. Assessing the cell viability after 72h of treatment allowed us to identify 11 validated hits (15.5% hit rate, calculated for the 71 virtual hits evaluated) that inhibit JAK1 dependent cell growth by at least 75% at 5µM concentration or lower. In addition, the cytotoxicities of the validated hits were determined, expressed as the residual viability of rat hepatocytes after exposure to the compounds. Cytotoxicity measurements were carried out by the Metabolic Drug Interactions Research Group at the Research Centre for Natural Sciences. This investigation confirmed that the activity of the compounds is not caused by general cytotoxicity.

The most represented chemotype of this set of JAK1 inhibitors is 8-hydroxy-quinoline, which is also a novel hinge-binding motif among publicly reported JAK inhibitors (Figure 2). We have followed up on this scaffold with a substructure- and similarity-based hit expansion from the Mcule database, ordering and testing ten further 8-hydroxyquinolines in the BaF3/JAK1\textsuperscript{V658F} cell-based assay, five of which have inhibited JAK1\textsuperscript{V658F}-driven cell growth with micromolar and submicromolar IC\textsubscript{50} (half-maximal inhibitory concentration) values. These final hit compounds have also been confirmed to be non-cytotoxic on rat hepatocytes. Interestingly, besides the common 8-hydroxy-quinoline scaffold, all of the compounds contain phenylpiperazine or benzylpiperidine moieties that suggests...
considering this core to be optimized in a subsequent medicinal chemistry program.

3.3 Virtual screening for inhibitors of wild-type JAK1

In our other effort to identify novel JAK1 inhibitors, we have virtually screened the Mcule Purchasable Compounds Database with a custom-made stepwise screening protocol that also involved the application of the Kinase Desirability Score as a pre-screening step.

Here, two JAK1 PDB structures were selected for docking from the (then) available fourteen: these two were later complemented with three frames from a 20ns-long all-atom MD simulation to provide further, diverse protein conformations for ensemble docking. The final protein ensemble was selected in a retrospective screening to optimize the early enrichment of known JAK1 inhibitors.

To discover novel JAK1 inhibitors, a prospective virtual screening was conducted with a stepwise screening protocol – including KiDS scoring and the optimized ensemble docking protocol – on the Mcule Purchasable Compounds Database, counting approx. 5.1 million compounds at the time. A set of 105,078 compounds (KiDS ≥ 4) was submitted to ensemble docking. From the ranked list of docked poses, we have selected the top ten compounds that were available for purchase at the time, and whose predicted binding modes could be verified by visual inspection (strong hinge-binding core, reasonable fitting into the ATP-pocket). These ten compounds were purchased from Mcule, and investigated in vitro against JAK1 in a Z’-LYTE enzyme-based kinase inhibition assay by Life Technologies. Five out of the ten compounds have been identified as confirmed hits, displaying micromolar and submicromolar IC$_{50}$ values against JAK1 (Table 2).
Table 2. Structures and JAK inhibition data of the resulting hit compounds

<table>
<thead>
<tr>
<th>#</th>
<th>Structure IC₅₀ (μM)ᵃ</th>
<th>Structure IC₅₀ (μM)ᵃ</th>
<th>Structure IC₅₀ (μM)ᵃ</th>
</tr>
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<tbody>
<tr>
<td></td>
<td><img src="image" alt="Structure B59" /></td>
<td><img src="image" alt="Structure B60" /></td>
<td><img src="image" alt="Structure B61" /></td>
</tr>
<tr>
<td>B59</td>
<td>JAK1: 0.558</td>
<td>JAK1: 1.680</td>
<td>JAK1: 0.433</td>
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<tr>
<td></td>
<td>JAK2: 1.710</td>
<td>JAK2: 0.648</td>
<td>JAK2: 0.514</td>
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<tr>
<td>B62</td>
<td><img src="image" alt="Structure B62" /></td>
<td><img src="image" alt="Structure B64" /></td>
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<tr>
<td></td>
<td>JAK1: 0.575</td>
<td>JAK1: 2.650</td>
<td></td>
</tr>
<tr>
<td></td>
<td>JAK2: 1.500</td>
<td>JAK2: 1.190</td>
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</table>

ᵃResults of 10-point titrations, where every data point was acquired twice. (Standard deviations were typically less than 10% of the mean.)

Three of the five hit compounds (those with submicromolar IC₅₀ values) define a new chemotype among JAK1 inhibitors, as they are all pyrrolopyrimidines carrying a spirocyclic substituent in the 4 position. Interestingly, these compounds display a moderate but clear preference for the JAK1 subtype (compared to JAK2). By calculating ligand efficiency metrics, we have also shown that they are suitable candidates for further medicinal chemistry optimization.
3.4 Virtual screening for subtype selective inhibitors of JAK2

Out of the many possible subtype selectivity profiles among JAK inhibitors, JAK2 vs. JAK1 selectivity is one of the hardest to achieve. In this study, we have addressed this issue and developed a customized virtual screening protocol for the identification of novel compounds with JAK2 vs. JAK1 subtype selectivity.

**Figure 3.** Summary of the prospective virtual screening. The early, property-based filters have considerably decreased the number of screened compounds, and thus the necessary computation time for the ensemble docking step. Docking, on the other hand, gives a more reliable prediction about those molecules that can establish favorable interactions.

The screening protocol and its retrospective validation is very similar to the one we have used for JAK1 inhibitors (see previous subsection and Figure 3), but in this case, the large amount of available training data has enabled us to develop a custom scoring method based
on interaction fingerprints (IFP) to predict JAK2 vs. JAK1 subtype selectivity. Together with the docking scores, the IFP score could be utilized to discriminate known JAK2 selective ligands (Figure 4).

**Figure 4.** Retrospective docking of JAK2 selective and reference compounds. In the plane spanned by IFP scores and Glide Docking Scores, an area can be separated that is populated exclusively by JAK2 selective compounds (bottom right). In the prospective screening, this area was utilized to select virtual hits.

After docking, 54 compounds were purchased from a diverse subset of the virtual hits, and 6 of them have been experimentally confirmed to inhibit JAK2. The indazole-based hit compound B39 has displayed a micromolar IC$_{50}$ value against JAK2 and 14-fold selectivity compared to JAK1. Three other micromolar inhibitors are also JAK2-selective (although this could not have been quantified). The hit compounds display favorable ligand efficiencies and B39 already constitutes the basis of an optimization study.
4. THESIS POINTS

1. I have developed a physicochemical property-based scoring scheme (KiDS – Kinase Desirability Score) for the rapid filtering of “kinase-like” compounds (ligands with a higher likeliness of kinase activity). I have demonstrated the applicability of this scoring scheme as a filtering step in structure-based virtual screening campaigns against kinase targets [T1].

2. I have set up a customized, step-wise virtual screening protocol for the identification of novel JAK1 inhibitors, involving a property-based filtering step with KiDS, and subsequent ensemble docking. A prospective screening of the Mcule database has yielded five novel JAK1 inhibitors – including three with a novel chemotype: spiro-substituted pyrrolopyrimidines –, displaying micromolar and submicromolar potencies [T2].

3. I have developed a customized virtual screening protocol for the identification of novel JAK2 inhibitors with subtype selectivity over JAK1, involving ensemble docking and a custom-developed scoring scheme for the prediction of subtype selectivity, based on interaction fingerprints. A prospective screening of the Mcule database has yielded six novel JAK2 inhibitors with micromolar activities and favorable subtype selectivities [T3].

4. I have identified five novel JAK1$^{V658F}$ inhibitors as a result of a virtual screening conducted on the NCI and Mcule databases. The five compounds bear a novel hinge-binding scaffold (8-hydroxyquinoline), are potent inhibitors of JAK1$^{V658F}$-driven cell growth, and have been demonstrated to be non-cytotoxic on rat liver cells [T4].
5. APPLICATIONS

The presented work can form the basis of various possible applications in the field of JAK-related drug discovery programs, some of which are already in progress in our research group.

The Kinase Desirability Score (KiDS) is a general tool that can be applied as a filtering step in any virtual screening campaigns that aim to identify novel kinase inhibitors. We have also published the basic principles that were applied during its development, which makes it possible to design similar scoring functions for other target classes as well.

Similarly, the interaction fingerprint scoring method that was developed for identifying subtype selective JAK2 inhibitors can be adapted for any pair of target and off-target proteins where sufficient training data are available, enabling the development of custom virtual screening protocols for compounds with a desired selectivity profile.

More generally, any step (or combination of steps) presented in our step-wise virtual screening protocols can be adapted in other works – for example the compilation of the protein ensembles for docking. The presented protocols have already served as a starting point for the development of virtual screening protocols for other types of JAK inhibitors in our research group – including type II inhibitors that bind to an inactive kinase conformation, as well as inhibitors that target the self-regulatory pseudokinase domain of Janus kinases.

Last but not least, we have shown that the new JAK1 and JAK2 inhibitors that were identified in our virtual screenings are promising candidates for medicinal chemistry optimization. In fact, the indazole-based JAK2 inhibitor B39 constitutes the basis of an ongoing optimization study in our research group.
6. PUBLICATIONS

6.1 Journal articles in the topic of the present thesis

T1. Property-based characterization of kinase-like ligand space for library design and virtual screening
D. Bajusz, G. G. Ferenczy, G. M. Keserű, Medicinal Chemistry Communications, 2015, 6, 1898-1904. IF: 2.319

T2. Ensemble docking-based virtual screening yields novel spirocyclic JAK1 inhibitors

T3. Discovery of subtype selective Janus kinase (JAK) inhibitors by structure-based virtual screening

T4. Identification of 8-Hydroxyquinoline Derivatives Active Against Somatic V658F Mutant JAK1 Dependent Cells

T5. Structure-based virtual screening approaches in kinase-directed drug discovery

6.2 Lectures and posters in the topic of the present thesis

1. Identification of novel, selective Janus kinase inhibitors with computational methods
D. Bajusz, G. G. Ferenczy, G. M. Keserű, Variations to four institutes, Budapest, Hungary, Nov 24, 2016 (in Hungarian)

2. Discovery of novel, potent inhibitors of Janus kinase 1 with virtual screening
3. Ensemble docking-based virtual screening reveals novel, subtype selective JAK2 inhibitors (poster)
4. Property-based characterization of ATP-site kinase inhibitor space for virtual screening (poster)
5. Virtual screening reveals novel Janus kinase inhibitors
6. A multi-step virtual screening approach for the identification of novel Janus kinase inhibitors
7. On the lookout for new drug candidates: virtual screening in drug discovery (guest lecture)
   D. Bajusz, Poznań University of Technology, Department of Organic Chemistry, Poznań, Poland, Sept 18, 2014
8. Desirability function-based scoring scheme for ATP-site kinase inhibitors (poster)
9. Large-scale screening of public chemical databases to identify novel selective Janus kinase inhibitors (poster)
10. Discovery of novel, selective Janus kinase inhibitors by virtual screening
11. Development of a virtual screening protocol to identify Janus kinase inhibitors
6.3 Journal articles and book chapters in other topics

1. Which performance parameters are best suited to assess the predictive ability of models?

2. Chemical data formats, fingerprints and other molecular descriptions for database analysis and searching

3. Chemoinformatics/Chemometrics in Analytical Chemistry

4. Multivariate assessment of lipophilicity scales—computational and reversed phase thin-layer chromatographic indices

5. Comparison of classification methods with “n-class” receiver operating characteristic curves: a case study of energy drinks

6. Consistency of QSAR models: ranking of models and performance parameters
A. Rácz, D. Bajusz, K. Héberger, SAR and QSAR in Environmental Research, 2015, 26, 683-700. IF: 1.897

7. Why is Tanimoto index an appropriate choice for fingerprint-based similarity calculations?

8. One vs. Two Electron Oxidation with Peroxomonosulfate Ion: Reactions with Iron(II), Vanadium(IV), Halide ions and Photoreaction with Cerium(III)