During the five year of the project we have conducted computational, synthetic chemistry and medicinal chemistry research, aiming for the identification and design of ATP-site JAK1 and JAK2 inhibitors and covalent JAK3 inhibitors. Since our studies aiming non-ATP-site JAK2 inhibitors were basically unsuccessful we initiated a new research line for the identification of compounds influencing downstream JAK/STAT signalling from the second year of the project. This work resulted in the concept of direct STAT inhibition and provided pharmacological tool compounds as the proof of the concept. Our research along the JAK/STAT pathway led to novel opportunities blocking JAK related signalling.

Main results are summarized in the following point with references:

1. We have developed a physico-chemical property-based scoring scheme (termed KiDS – Kinase Desirability Score) for filtering large molecular databases, to enrich so-called "kinase-like" ligands (molecules with an increased likeliness to display inhibitory activity on kinases). The scoring method utilizes the concept of desirability functions and accounts for six whole-molecular descriptors, hence it allows for a greater diversity of ligands to be selected, than e.g. 2D fingerprints. The method was validated on several independent datasets and was shown to be efficient in the enrichment of known kinase inhibitors. [1]
2. We have conducted a virtual screening campaign on the Mcule database to identify ATP-site JAK2 inhibitors that are subtype selective for JAK2 (vs. JAK1). We have applied the already mentioned KiDS scoring scheme as a pre-filtering step, followed by an ensemble docking protocol, based on a total of five crystallographic and MD-simulated JAK2 structures. The docking results were complemented with a custom-developed interaction fingerprint (IFP) based scoring scheme that was trained to distinguish known compounds with JAK2 vs. JAK1 subtype selectivities. Based on the docking scores and IFP scores, 429 virtual hits were identified and further filtered based on diversity and visual inspection. Finally, 54 compounds were purchased, six of which have been experimentally confirmed in an enzyme-based JAK1 inhibition assay (Life Technologies). Of these hit compounds, the indazole-based inhibitor B39 have displayed low micromolar inhibition against JAK2 and a 14-fold preference for JAK2 over JAK1. Three other hit compounds have displayed single-digit micromolar inhibitory potencies against JAK2 and negligible inhibition of JAK1 at a concentration of 20μM, suggesting favourable subtype selectivities as well. [2]
3. We have compiled a comprehensive review of the literature on structure-based virtual screening against kinase targets [3].
4. We developed a sequential virtual screening protocol to identify ATP-site JAK1 inhibitors. The protocol involved the previously developed KiDS desirability scoring scheme that prioritizes kinase inhibitor like compounds. Applying KiDS against a commercial compound library (Mcule Purchasable Compounds Database) with a customized screening protocol that involved the KiDS scoring scheme as a pre-filtering step, followed by ligand docking to crystallographic and MD-simulated JAK1 structures. Our virtual screening efforts were followed up by testing 10 virtual hits experimentally, out of which five have displayed single-digit micromolar and submicromolar IC50 values on JAK1. The results have highlighted spirocyclic pyrrolopyrimidines with submicromolar JAK1 IC50 values and a preference for JAK1 over JAK2 as potential starting points in developing a novel class of JAK1 inhibitors. [4]
5. We developed a pharmacophore driven docking protocol for the identification of JAK1 inhibitors that are active on V658F somatic mutant cells. We screen the National Cancer Institute (NCI) database by property-based pre-filtering steps and ligand docking to a

crystallographic JAK1 structure with optimized pharmacophore constraints. The screening has yielded five new, experimentally validated inhibitors of JAK1 with 8-hydroxyquinoline as a novel hinge-binding scaffold. The compounds have not only displayed favourable potencies in a JAK1V658F-driven cell-based assay, but were also shown to be non-cytotoxic on rat liver cells. [5]

6. Our group joined to a research programme led by Richard Moriggl from the University of Veterinary Medicine and the Ludwig Boltzmann Institute for Cancer Research, in collaboration with the group of Patrick Gunning at the University of Toronto, which aims to renew the therapeutic options for the treatment of acute myeloid leukemia (AML). The JAK/STAT signalling pathway is a central core cancer pathway that has a key role in this leukemic processes and it is implicated in other myeloproliferative diseases as well as many other cancer types. The somatic V617F mutation of JAK2 renders the tyrosine kinase, which is acting like an enzyme, super-active promoting strong activation of STAT5, a key downstream transcription factor that pushes into oncogene induction. Therefore, in addition to the JAK2 inhibitors aimed in this project, STAT5 inhibitors might contribute significantly to block oncogenic signalling in AML. We investigated this opportunity and identified interconnections between the JAK-STAT pathway, epigenetic regulation or DNA damage control. Analysing the mutational landscape of myeloproliferative neoplasms and peripheral T-cell leukemia and lymphomas we found similar combinations of driver mutations. This finding supports that these pathways might be interconnected in normal or cancer cells, which have lost differentiation capacity and drive oncogene transcription. Consequently, elements of the JAK/STAT signalling pathway can be considered as promising drug targets in multiple myeloproliferative diseases. These findings has been published in Exp. Opin. Ther. Pat. [6].
7. Joining forces between Hungary, Austria and Canada we reported a new, efficacious STAT5 inhibitor, which has successfully blocked cell division and growth in AML cell lines, as well as animal models. Clinical relevance of the results has been verified in human cells, from leukemia patients. Our research group have contributed in the design phase when exploring the molecular mechanism of the process. Following STAT phosphorylation, two STAT proteins form a parallel dimer, which then rapidly goes nuclear to bind to DNA in the cell nucleus to initiate gene transcription. However, too much STAT5 drives cancer through oncogene induction promoting cellular growth and survival. The new inhibitor is capable of blocking dimer formation, via binding to the SH2 domain of STAT5, rendering the protein unable to bind to DNA and to start oncogene transcription. Direct inhibition of STAT5 through the developed compound can represent a new therapeutic opportunity not only in leukemia, but hopefully also in other oncological diseases where STAT signalling is of central importance. Clinical development of the compound with structural modelling and further chemical modification and fine tuning in with testing in animal models in Austria, Hungary and Canady can lead in the near future to better answers how to battle AML. These results have been published in the highly recognized Nature group journal, Leukemia [7].
8. During the optimization of B39, a low micromolar inhibitor of JAK2 showing preference over JAK1, we identified three regions of specific interest. One is the optimal substitution pattern of the hinge binding indazole core, the second is the

linker between this core and the distant aromatic moiety of B39, and the third is the substitution pattern of the distant aromatic core located in the selectivity pocket of JAK2. Combining the beneficial changes identified previously at each region, however, we were unable to identify novel B39 analogues with improved potency and pharmacological profile. Interestingly we found, that there is a significant crosstalk between the hinge binder and the distant aromatic core. Since the gain in the potency was limited with the less flexible heterocyclic linkers, we used the amide linker and optimized both aromatic moieties thoroughly. During this work we prepared 15 new analogues having the original indazole and the modified 3-amino-indazole core responsible for hinge binding and phenyl ring with diverse substituents (heterocyclic, N-alkyl and halogens) as a distant aromatic core. Carefully optimizing the substituents at the hinge binding region and in the selectivity pocket we identified a compound with improved submicromolar affinity and JAK2/JAK1 selectivity. Structure-activity relationship (SAR) analysis revealed the importance of structural waters and their networks that influence both the activity and selectivity of B39 derivatives. Investigating the binding mode of these derivatives in the JAK2 pocket suggests that binding site waters play a crucial role in the activity-selectivity trade-off of this set of JAK2 inhibitors. [8]

9. We explored the potential binding sites on a large number of protein kinases. While the locations of the sites that bind type II and III inhibitors at or near the adenosine 5'-triphosphate binding sites are well defined, the literature describes 10 different regions that were reported as regulatory hot spots in some kinases and thus are potential target sites for type IV inhibitors. Kinase Atlas is a systematic collection of binding hot spots located at the above ten sites in 4910 structures of 376 distinct kinases available in the Protein Data Bank. We identified the potential binding hot spots by FTMap and made them available as an online resource named Kinase Atlas. Users of Kinase Atlas (https://kinase-atlas.bu.edu) may view summarized results for all structures of a particular kinase, such as which binding sites are present and how druggable they are. This would facilitate exploring the available binding sites experimentally and might help generating potent and selective allosteric modulators for therapeutically relevant kinases. [9]
10. We developed a conceptually new fragment library consisting 84 electrophilic small heterocycles. The library was characterized through cysteine-reactivity and aqueous stability tests that suggested their potential as covalent warheads. The analysis of theoretical and experimental descriptors revealed correlations between the electronic properties of the heterocyclic cores and their reactivity against GSH that are helpful in identifying suitable fragments for cysteines with specific nucleophilicity. The most important advantage of these fragments is that they show only minimal structural differences from non-electrophilic counterparts. Therefore, they could be used effectively in the design of targeted covalent inhibitors with minimal influence on key non-covalent interactions. This work has been first communicated at the RSC FRAGMENTS 2019 meeting in Cambridge and was picked up by the FBDD community and by the Practical Fragments blog (http://practicalfragments.blogspot.com/2019/06/new-chemistries-for-covalent-fragments.html). It is now used at the XChem facility of Diamond Light Source, Oxford for covalent X-ray screening against different targets. GlaxoSmithKline and further academic collaborators are also evaluating the library in different screening

programs. More recently, we signed a commercialization agreement with ComInnex, the company responsible for the marketing and distribution of the library for commercial partners (https://cominnex.com/libraries/).

11. Covalent inhibitors consist of two key elements, one is the electrophilic warhead that forms covalent interaction with an appropriate nucleophilic residue of the target protein, and the other is a scaffold that forms exclusively non-covalent interactions within the same binding pocket. Other than targeting non-conserved nucleophilic residues, optimizing this noncovalent binding framework is important to improve potency and selectivity of covalent binders toward the desired target. To highlight the value of the noncovalent complex in the covalent binding process, we developed a new computational protocol using tethered and constrained docking in combination with Dynamic Undocking (DUck) as a tool to privilege strong protein binders for the identification of novel covalent inhibitors. At the end of the protocol, dedicated covalent docking methods were used to rank and select the virtual hits based on the predicted binding mode. The power of this methodology has been demonstrated in different targets including JAK3. Starting from a large set of commercially available acrylamides, a prototypic set of covalent inhibitors we prioritized 10 compounds using DUckCov. Testing the compounds in the relevant JAK3 assay, three of them showed considerable JAK3 inhibitory property. One of these compounds has an IC50 of 5 nM and showed at least 100-fold selectivity over JAK1 and JAK2. This work was highlighted on the front cover of the Journal (https://onlinelibrary.wiley.com/doi/10.1002/cmdc.201900263).
12. Although a number of computational protocols have been published for identifying druggable cysteines in protein kinases, experimental approaches are limited. In this work we developed a toolbox of fragment-sized molecules containing an identical scaffold but equipped with diverse covalent warheads covering a range of electrophilic properties. Screening this library against multiple kinases could experimentally characterize the accessibility and reactivity of the targeted cysteine and might help to identify suitable warheads for designed covalent inhibitors as exemplified retrospectively and prospectively for JAK3 inhibitors. Combining preselected covalent warheads identified from the warhead library with the known noncovalent hinge-binding scaffold (4-phenyl-pyrrolopyrimidine) yielded potent and selective JAK3 inhibitors. The impact of binding site waters on the activity/selectivity trade-off of Janus kinase 2 (JAK2) inhibitors. [12]
13. We reviewed the recent principles and considerations in the design of electrophilic fragment libraries from the selection of the appropriate covalent warhead through the design of the covalent fragment to the compilation of the library. Recent screening methodologies of covalent fragments against surrogate models, proteins, and the whole proteome, or living cells were also summarized. Finally, we highlighted recent drug discovery applications of covalent fragment libraries that provide case studies for upcoming covalent drug discovery programs.
14. In signal transducer and activator of transcription (STAT) proteins, SH2 domain interactions are critical for molecular activation and nuclear accumulation of phosphorylated STAT dimers to drive transcription. Interestingly, mutations occured in these domains are implicated in a high number of cancers, however, structural data regarding the distinctive STAT-type SH2 domain is limited. Here, we review the unique features of STAT-type SH2 domains in the context of all currently

reported STAT3 and STAT5 SH2 domain clinical mutations. The genetic volatility of specific regions in the SH2 domain can result in either activating or deactivating mutations at the same site in the domain, underscoring the delicate evolutionary balance of wild type STAT structural motifs in maintaining precise levels of cellular activity. Understanding the molecular and biophysical impact of these disease-associated mutations can uncover convergent mechanisms of action for mutations localized within the STAT SH2 domain to facilitate the development of targeted therapeutic interventions [14].

15. Signal transducer and activator of transcription STAT3 and STAT5 are important transcription factors that are able to mediate or even drive cancer progression through the JAK/STAT signalling pathway. Classical understanding of STAT functions is linked to their phosphorylated parallel dimer conformation, in which they induce gene transcription. However, the functions of STAT proteins are not limited to their phosphorylated dimerization form. In our review [15], we discuss the functions and the roles of unphosphorylated STAT3/5 and analyse these proteins as potential drug targets. Although currently there are no direct STAT3/5 inhibitors of clinical grade available, we summarize the development of inhibitors against the SH2 domains of STAT3/5 and discuss their clinical potential.

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