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NOVEL TARGETED INHIBITORS FOR JAK1 AND JAK3 TYROSINE KINASES

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ABSTRACT

Kirsikka Musta: Novel targeted inhibitors for JAK1 and JAK3 tyrosine kinases
Master's Thesis
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Janus kinases (JAKs) are a family of tyrosine kinases that are important in inflammation. Janus kinase inhibitors are used to treat autoimmune and inflammatory diseases like rheumatoid arthritis and inflammatory bowel disease.

JAK activity is mediated by the tyrosine kinase domain (JH1) which is regulated by the pseudokinase domain (JH2). All the clinical JAK inhibitors on the market target the JH1, which is more conserved than JH2. Most of the current clinical JAK inhibitors are not specific to one JAK but instead inhibit the activity of many or all of the JAKs. The aim of this thesis was to find an inhibitor, that inhibits the JAK activity by binding to JH2 domain instead. This way the inhibition could be specific to either JAK1 or JAK3, reducing side-effects by better targeting the cytokine signalling that is inhibited.

In this thesis, a group of potential JH2 binders were investigated. Some of them were hits from previous smw screenings, but some were identified through a virtual screen and characterized as part of this thesis. The potential JH2 binders for JAK1 or JAK3 were studied fortheir binding specificity and inhibition. Inhibition was studied by Lance Ultra, which analysed the JH1 kinase activity in the context of JH2 regulation, and by cytokine signalling studies which analysed the inhibition of downstream signalling in cellular context.

Three compounds, Omipa, Picti and 4G were identified as potential JAK1 JH2 specific binders, and two compounds, Mito and F165 also showed TYK2 JH2 binding along with JAK1 JH2 binding. The compounds did not show inhibition in kinase activity assay but did show inhibition of downstream signalling of either IL6 or IL4. Due to technical issues in cytokine signalling assay, these results need to be replicated in order to validate the findings. The assay could be improved by optimizing the freezing process of the cells or by using unfrozen cell and by optimizing fluorescence barcoding.

Keywords: Janus kinase, JAK, JAK inhibitor, JAKinib, JAKinibs, Rheumatoid Arthritis, RA

TIIVISTELMÄ

Kirsikka Musta: Uudet kohdennetut inhibiittorit JAK1:lle ja JAK3:lle
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Janus kinaasit (JAK) ovat tyrosiinikinaasi perhe jotka ovat tärkeitä tulehduksessa. Janus kinaasien inhibiittoreita käytetään autoimmuuni- ja tulehduksellisten sairauksien kuten nivelreuman ja tulehduksellisten suolistosairauksien hoitoon.

JAK aktiivisuutta välittää tyrosiinikinaasi osa (JH1) jota säätelee pseukokinaasidomeeni (JH2). Kaikki markkinoilla olevat kliiniset JAK inhibiittorit sitoutuvat JH1-osaan joka on konservoituneempi kuin JH2i. Useimmatkäytössä olevat kliiniset JAK inhibiittorit eivät ole spesifisiä yhdelle JAK:ille, vaan inhiboivat monien tai kaikkien JAK:ien aktiivisuutta. Tämän opinnäytetyön tavoiteena oli löytää inhibiittori, joka inhiboi JAK aktiivisuutta sitoutumalla JH2 osaan. JH2 välitteinen inhibitio voisi olla spesifistä joko JAK1:lle tai JAK3:lle, vähentäen sivuvaikutuksia kohdentamalla paremmin inhiboitua sytokiinisignalointia.

Tässä opinnäytetyössä tutkittiin ryhmää potentiaalisia JH2 sitoutuja. Osa niistä oli löydöksiä aiemmista virtuaaliseulonnoista, mutta osa tunnistettiin virtuaalisessa seulonnassa, joka on sisällytetty tähän opinnäytetyöhön. Potentiaalisista JAK1 tai JAK3 JH2 sitoutujista tutkittiin sitoutumisen spesifisyys ja inhibitio. Inhibition tutkittiin käyttämällä Lance Ultraa, mikä tutkii JH1:n kinaasiaktiivisuutta yhdessä JH2:n säätelyn kanssa. Inhibitiota tutkittiin myös sytokiinisignalointia tutkimalla, mikä tarkastelee solusignaloinnin inhibitiota.

Kolme yhdistettä, Omipa, Picti ja 4G tunnistettiin potentiaalisiksi JAK1 JH2 spesifisiksi sitoutujiksi ja kaksi yhdistettyä, Mito ja F165 osoittivat lisäksi myös TYK2 JH2 sitoutumistai. Nämä yhdisteet eivät osoittaneet inhibitiota kinaasiaktiivisuuden tarkastelussa, mutta osoittivat joko IL6:n tai IL4:n alavirran signaloinnin inhibitiota. Johtuen teknisistä ongelmista sytokiinisignalointikokeissa, nämä tulokset tulee toistaa löydösten validoimiseksi. Sytokiinisignalointikoetta voisi kehittää optimoimalla solujen jäädytystä tai käyttämällä tuoreita soluja sekä optimoimalla fluoresenssi barkoodausta.

Avainsanat: Janus kinaasi, JAK, JAK inhibiittori, Nivelreuma

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

FP	Fluorescence polarization
FSC	Forward scatter
HTS	High throughput screening
IBD	Inflammatory bowel disease
IL	Interleukin
JAK	Janus kinase
PBMC	Peripheral blood mononuclear cells
RA	Rheumatoid Arthritis
SEC	Size-exclusion chromatography
SSC	Side scatter
STAT	Signal transducer and activator of transcription

1 INTRODUCTION

Janus kinases (JAKs) and signal transducer and activator of transcription (STATs) play an essential role in cytokine signalling and they are promising targets for new drugs to treat different autoimmune and inflammatory disorders. This master's thesis research focuses of JAK1 and JAK3 but there are also two additional JAKs: JAK2 and TYK2. JAKs have four domains: FERM, SH2, pseudokinase JH2 and finally active kinase domain JH1.

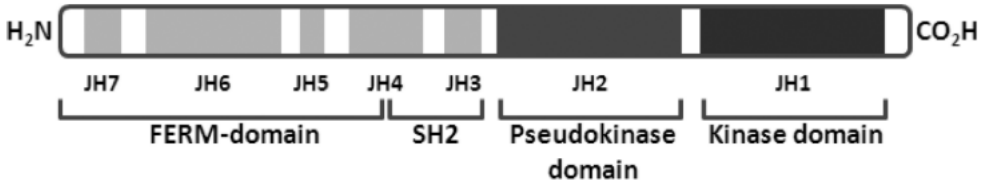

Figure 1. *JAK domains, figure adopted from* (Clark et al., 2014)

The pseudokinase JH2 domain has an essential role in the regulation of JH1 domain. When there is no stimulation JH2 inhibits the activation of JH1 domain and thus its autophosphorylative function but when a signal does arrive, JH2 mediates the signal from the receptor to the JH1 domain (Hammarén, Virtanen, Raivola, et al., 2019; Saharinen et al., 2000; Saharinen & Silvennoinen, 2002). Furthermore, JH2 binds ATP (Hammarén et al., 2015; Raivola et al., 2018). All this makes JH2 an interesting target for inhibition. Diseases where JAK1 and/or JAK3 inhibition might be beneficial include but are not limited to Rheumatoid Arthritis (RA), Inflammatory bowel disease (IBD) and Psoriasis as well as different Leukaemia and cancers where JAK 1 or 3 is hyperactive by mutations (Virtanen et al., 2019). There already are nine JAK inhibitors on the market: tofacitinib, baricitinib, ruxolitinib, filgotinib, upadacitinib, peficitinib (Jegatheeswaran et al., 2019), fedratinib (Gadina et al., 2018), delgocitinib (Dhillon, 2020) and abrocitinib (Deeks & Duggan, 2021) but they all target the JH1 domain (Wrobleski et al., 2019a).

JH2 domain might be a better target to an inhibitor since JH1 domain has an ATP-binding pocket that is very conserved, making it difficult to have specificity (Banerjee et al., 2017). JH2 domain is also a known hotspot for different illness causing mutations. (Virtanen et al., 2019) These factors mean that JH2 domain potential sites that could be used as targets and since the sites are not as conserved as JH1 ATP binding site, it would be possible to introduce higher level of specificity.

2 LITERATURE REVIEW

2.1 Introduction to JAKs

JAK-STAT signalling is important part of cell signalling. It takes part in fundamental processes like cell proliferation, apoptosis and cytokine signalling (Xin et al., 2020). It is observed in a many species (Bousoik & Montazeri Aliabadi, 2018) and it has existed for over 500 million years (Banerjee et al., 2017). There are four different JAKs (JAK1, JAK2, JAK3 and TYK2) and seven different STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6) (Bousoik & Montazeri Aliabadi, 2018).

2.1.1 JAK/STAT pathway

JAK-STAT signalling starts when a signalling molecule binds to a receptor (Murray, 2007). Receptors used by JAK/STAT signalling pathway include cytokine receptors, G-protein coupled receptors, growth factor receptors and receptor tyrosine kinases, but cytokine receptors are the most common of these (Bousoik & Montazeri Aliabadi, 2018). Cytokine receptors use tyrosine kinases like JAKs because some cytokine receptors lack the needed kinase activity to relay extracellular signals to the nucleus (Clark et al., 2014).

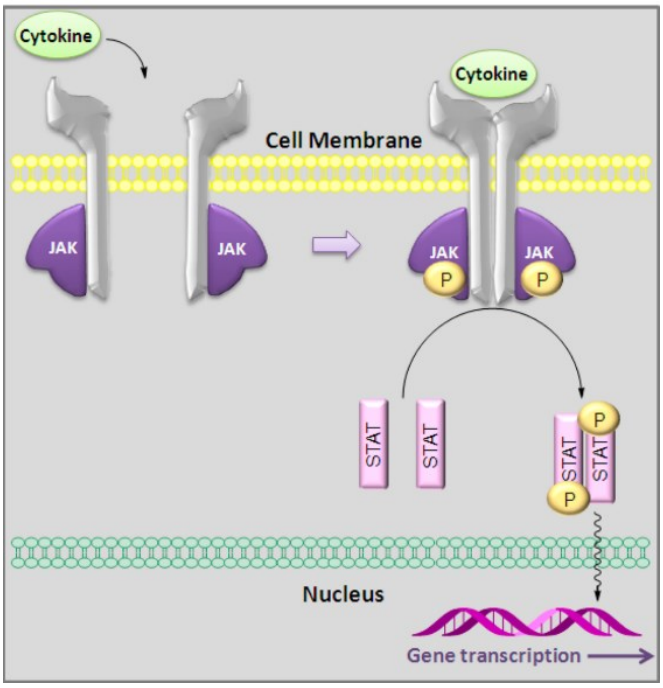

Figure 2. *JAK/STAT pathway. Binding of a cytokine into a cytokine receptor causes JAKs to activate. The activated JAKs recruit STATs, which are phosphorylated and act as transcription factors in the nucleus. Adopted from* (Clark et al., 2014)

The binding of a cytokine into a cytokine receptor changes the conformation of the associated intracellular subunits of the cytokine receptor, which allows JAKs to activate

(Banerjee et al., 2017). This causes the cytoplasmic JAKs to autophosphorylate and become active (Dodington et al., 2018). The activated JAKs recruit STAT, which are then phosphorylated and form active homodimers, heterodimers or tetramers (Banerjee et al., 2017). STATs, which are transcription factors, then locate to the nucleus to bind a specific DNA sequence to activate gene expression (Murray, 2007).

There are many kinds of JAKs and STATs. There are 4 JAKs: JAK1, JAK2, JAK3 and TYK2 (Xin et al., 2020). They work together in different combinations, which makes it possible to amplify the number of signals JAKs can transduce (Clark et al., 2014). The possible dimers and oligomers are depicted in Figure 3. Table 1 shows the cytokine receptors, many of which are interleukins (ILs), associated with each JAK dimer or oligomer. Although IL-3 signals mainly though JAK2/JAK2 dimer, studies have shown that JAK1 plays a role in some way, because cells deficient in JAK1 show reduced IL-3 signalling (Dougan et al., 2019).

Figure 3. *Different JAK dimers and oligomers,* modified from Clark et al., 2014

Table 1. *JAK dimers and cytokine receptor.*
Based on (George Abraham et al., 2020) and (Dougan et al., 2019)

JAK dimers/oligomers	***Cytokines***
JAK1/JAK2	IL-19, IL-35, IFNγ
JAK1/JAK3	IL-2, IL-4, IL-7, IL-9, IL-15, IL-21
JAK1/TYK2	IL-10, IL-22, IL-26, IL-28, IFNα, IFNβ
JAK1/JAK2/TYK2	IL-6, IL-11, IL-13, IL-27, LIF
JAK2/JAK2	IL-3, IL-5, GM-CSF, EpoR, GHR, PRL, TpoR
JAK2/TYK2	IL-12, IL-23

2.1.2 JAK activation

In order to mediate signals, JAKs need to be activated. JAK activity is regulated in a complex manner. In the centre of JAK regulation lies JH2 domain, which is called pseudokinase due to the fact that it lacks kinase activity (Hammarén, Virtanen, Raivola, et al., 2019). This regulatory role is highlighted by the fact that vast majority of activating mutations are located in the JH2 domain (Babon et al., 2014).

JH2 has a dual role as an inhibitor and as an activator (Hammarén, Virtanen, Abraham, et al., 2019; Raivola et al., 2018; Saharinen & Silvennoinen, 2002; Ungureanu et al., 2011). When there is no signal from the receptor, JH2 works as an inhibitor to the JH1 kinase domain, preventing its activation (Babon et al., 2014). In experimental studies it has been shown that, JAK2 JH1-2 domain is 50 less active than JAK2 JH1 domain alone (Roskoski, 2016). The inhibition is likely done by blocking JH1 from achieving the conformational changes needed for kinase activity (Hammarén, Virtanen, Raivola, et al., 2019) There are two proposed models for JH2 mediated inhibition of JH1: it could happen *in cis* or *in trans* (Brooks et al., 2014; Ungureanu et al., 2011). Cis-model proposes that JH2 inhibits the JH1 of the same JAK protein intramolecularly, while trans-model proposes that JH2 inhibits the activation of the other JAK intermolecularly (Roskoski, 2016). It is not clear, which model is being used, but there is experimental data supporting both models (Babon et al., 2014).

JAK activation starts when a receptor relays the activation signal to the JH2 pseudokinase, which then mediates the activation signal to the JH1 domain from the receptor (Hammarén, Virtanen, Raivola, et al., 2019).

2.2 Role of JAKs in autoimmune and inflammatory disorders

JAK/STAT pathway has been connected to the pathogenesis of autoimmune and inflammatory diseases (Banerjee et al., 2017). These diseases include but are not limited to rheumatoid arthritis (RA), psoriasis and inflammatory bowel diseases (IBD) (Roskoski, 2016). They are characterized by immune reaction targeting the body's own tissue which leads to dysfunction or damage to tissues (Ngo et al., 2014).

In autoimmune and inflammatory diseases, cytokines play a huge role in mediating the immune reaction. The cytokine profile varies from disease to disease (Clark et al., 2014). Different JAKs dimers and oligomers respond to different cytokines, as was shown on Table 1. This means that there is a possibility that targeting the JAK specifically active in the disorder would be therapeutically beneficial while reducing side effects that inhibitors that inhibit JAKs more broadly.

2.2.1 Role of JAKs in RA

Rheumatoid arthritis (RA) is an autoimmune disorder where the immune system targets the tissue of the joints (Martio et al., 2007). The inflammation can present via joint stiffness, pain and swelling and sometimes also warmth (Watts et al., 2013). The joints typically affected are small joints in hands and wrists as well as feet (Zhang & Lee, 2018). Damage to the synovial joints and increased likelihood of disability are also possible (McInnes et al., 2016). The disease can enter a state of remission where there are no symptoms present, which is especially made possible by modern treatment (Watts et al., 2013).

It is not known what causes RA, but genetics plays a role (Firestein & McInnes, 2017) as well as environmental factors such as smoking and socio-economic status

(Watts et al., 2013). RA affects about 0.3 to 4.2% of the people, depending on the population (Zhang & Lee, 2018) and the average age on onset is 60 years old (Martio et al., 2007).

RA is commonly treated with methotrexate, but corticosteroids can also be used (Ngian, 2010). JAK inhibitors tofacitinib, baricitinib (Firestein & McInnes, 2017), upadacitinib (Rocha et al., 2021) and filgotinib (E. S. Kim & Keam, 2021) as well as peficitinib in Japan has been approved for the treatment of RA (Rocha et al., 2021).

In RA several key cytokines drive the inflammation. The cytokine profile is likely different in the onset of the disease as compared to established disease (McInnes et al., 2016). Notable cytokines in RA include IL6, tumour necrosis factor alpha (TNFα), several members of IL1 family and granulocyte-macrophage colony-stimulating factor (GM-CSF) (McInnes et al., 2016). The importance of TNFα is highlighted in the fact that biological anti-TNFα medication is used to treat RA (Martio et al., 2007). More cytokines that have been implicated in RA pathogenesis have been depicted in Table 2.

Table 2. *Some cytokines relevant in RA and the JAKs they signal through*

Cytokines relevant in RA	***JAKs related to the receptors***
GM-CSF[1]	JAK2/JAK2[3]
IL-6[1]	JAK1/JAK2/TYK2[3]
IL-17[1]	Doesn't signal through JAK/STAT pathway[4]
IL-12[1]	JAK2/TYK2[3]
IL-23[1]	JAK2/TYK2[3]
IL-4[2]	JAK1/JAK3[5]
IL-7[2]	JAK1/JAK3[5]
IL-15[2]	JAK1/JAK3[5]
IL-21[2]	JAK1/JAK3[5]

[1](McInnes et al., 2016)
[2](Virtanen et al., 2019)
[3](Banerjee et al., 2017)
[4](Gaffen, 2009)
[5](George Abraham et al., 2020)

As can be seen from the table 2, there are several cytokines relevant to the RA that signal through JAK1 and/or JAK3. These include IL-6, IL-4, IL-7, IL-15, and IL-21. This means that selective inhibition of JAK1 or JAK3 could have therapeutic effects in the treatment of RA.

2.3 Malignancies

Gain of function mutations of JAKs have been reported in different kinds of malignancies (Gadina et al., 2018). In fact, different JAK mutations have been reported in hematopoietic malignancies, leukaemia, lymphomas and cancer (Hammarén, Virtanen, Raivola, et al., 2019). These mutations are often reported in the JH2 domain (Hammarén, Virtanen,

Raivola, et al., 2019). For example V617F mutation in JAK2 causes JAK2 to be activated in several myeloproliferative neoplasms (Clark et al., 2014). In fact, there are hyperactive mutations of JAK1 and JAK3 linked to different forms of leukaemia that have been identified. For JAK1 notable mutations include V623A, S646P and V658F while for JAK3 M511I, A573V and R657Q have been identified (Raivola et al., 2021). Furthermore, for acute megakaryoblastic leukaemias, mutations A572V, V772I and P132T have been detected for JAK3 (Jeong et al., 2008).

For these reasons JAK inhibitors are being studied for assortment of different solid organ cancers and haematological malignancies (Gadina et al., 2018). For example ruxolitinib has been approved for the treatment of myelofibrosis (Roskoski, 2016). This means that the JAK inhibitors studied in this thesis have potential to also be used as a treatment for malignancies.

2.4 Existing JAK inhibitors

There are JAK inhibitors on the market already, and they are presented in Table 3. The nine JAK inhibitors on the market for humans are: tofacitinib, ruxolitinib, baricitinib (Banerjee et al., 2017), peficitinib, fedratinib, upadacitinib (Gadina et al., 2020), filgotinib (Jegatheeswaran et al., 2019), delgocitinib (Dhillon, 2020) and finally abrocitinib (Deeks & Duggan, 2021). They can be further divided into first-generation JAK inhibitor and second-generation JAK inhibitors based on selectivity. Fist-generation JAK inhibitors inhibit JAKs more broadly and, but second generation JAK inhibitors aim to be more selective to reduce side effects (Gadina et al., 2018).

Table 3. *Licensed JAK inhibitors and the JAKs they inhibit*

JAK inhibitor	***Targeted JAK***	***Selectivity***
Tofacitinib	JAK1/3 (and JAK2)[1]	Non-selective[5]
Ruxolitinib	JAK1/2[1]	Non-selective[3]
Baricitinib	JAK1/2[1]	Non-selective[5]
Peficitinib	JAK1/3[1]	Non-selective[7] or selective[1]
Fedratinib	JAK2[2]	Selective[6]
Upadacitinib	JAK1[3]	Selective[5]
Delgocitinib	Pan-JAK[4]	Non-selective[4]
Filgotinib	JAK1[1]	Selective[5]
Abrocitinib	JAK1[8]	Selective[8]

[1] (Winthrop, 2017)
[2] (Xin et al., 2020)
[3] (Gadina et al., 2018)
[4] (Dhillon, 2020)
[5] (H.-O. Kim, 2020)
[6](Roskoski, 2016)
[7](Markham & Keam, 2019)
[8](Deeks & Duggan, 2021)

2.4.1 The philosophy of selectivity

It is sometimes difficult to establish the JAKs a JAK-inhibitor inhibits. For example, it has been reported that peficitinib is a Pan-JAK inhibitor that binds JAK1, JAK2, JAK3 and TYK2 (Markham & Keam, 2019), but it has also been reported that peficitinib is a JAK1/3 inhibitor (Winthrop, 2017). It can be said that peficitinib has moderate selectivity to JAK3 while also inhibiting other JAKs (Takeuchi et al., 2016).

This is because it is not fully established where the line of selectivity is. Even selective JAK-inhibitors have some potential to inhibit other JAKs. This can be seen in Table 4, where the IC_{50} values of different inhibitors towards different JAKs are compared. IC_{50} refers to half of maximum inhibitory concentration and it is commonly used to describe inhibition.

Table 4. *Enzyme activity inhibition IC_{50} values*

Inhibitor	***JAK1 IC_{50} (nM)***	***JAK2 IC_{50} (nM)***	***JAK3 IC_{50} (nM)***	***TYK2 IC_{50} (nM)***	***Citation***
Peficitinib	3.9	5.0	0.71	4.8	(Markham & Keam, 2019)
Filgotinib	10	28	810	116	(Virtanen et al., 2019)
Upadacitinib	8	600	2300	NA	(Virtanen et al., 2019)
Tofacitinib	3.2	4.1	1.6	34.0	(Virtanen et al., 2019)

Table 4 shows, that when the IC_{50} values for the inhibition of each JAK are compared between peficitinib and selective filgotinib and upadacitinib, it is revealed that even though both filgotinib and upadacitinib are selective to JAK1, filgotinib shows some inhibition of JAK2. The table also shows why peficitinib can be considered Pan-JAK inhibitor, since the level of inhibition is quite high for all the JAKs, even if the inhibition is the strongest towards JAK1 and especially JAK3.

Additionally, tofacitinib is sometimes referred to as a pan-JAK inhibitor (H.-O. Kim, 2020) and other times as a JAK1/3 inhibitor (Winthrop, 2017). It can similarly be seen on the table that tofacitinib also inhibits JAK2 well. By inhibiting three out of four JAKs well, it can be argued that tofacitinib is not a selective inhibitor. The reason for the fact that tofacitinib is sometimes considered a selective inhibitor might be because it has been reported that in high doses tofacitinib inhibits other JAKs more easily (Winthrop, 2017). This means that in smaller doses tofacitinib is more selective. All of this goes to show how hard it can be to draw the line with selectivity even with clinically used inhibitors.

The situation is complicated by the fact that different assays can give different IC_{50} values as results. This is partially because binding affinity does not always correlate with inhibitory potential of a compound (Xu et al., 2016). This means that even if binding affinity assays establish an IC_{50}-value, the actual inhibition IC_{50} value from activity inhibition

assay might be different. Furthermore, some assays simple give different results. For example, when tofacitinib was being researched, solid-phase ELISA assays indicated that tofacitinib has 100-fold specificity on JAK3 over JAK1. However, when the selectivity was studied using Caliper assay, it was revealed that tofacitinib was not selective to JAK3 after all. (Flanagan et al., 2014) Moreover, the effect a compound has on signalling inhibition might also diverge from the binding affinity and inhibition of activity. In the case of JAKs this can be due to the dominant nature of one JAK in a JAK heterodimer, which makes it difficult to inhibit the cytokine signalling through the less dominant JAK (Virtanen et al., 2019). All this means that when comparing the IC_{50} values for JAK inhibitors, how the IC_{50} value has been studied also needs to be taken into consideration.

The reason higher selectivity regarded as a good thing is that it is hoped that this was side effects could be reduced while not sacrificing the effectiveness. Side effects for non-selective Tofacitinib and Baricitinib include infections and malignancies (H.-O. Kim, 2020). Selectivity can reduce side effects experienced by the patients (Moodley et al., 2016).

2.4.2 JAK inhibitors that target JH2 domain

Most JAK inhibitors target the active site of JH1 domain (Wrobleski et al., 2019b). Deucravacitinib is the first inhibitor known to bind JH2 to enter clinical trials (Wrobleski et al., 2019b). Deucravacitinib is also known as BMS-986165 (Jo et al., 2021) and inhibits TYK2 allosterically (Wrobleski et al., 2019b). This means that currently there are no clinically accepted inhibitors that bind to JH2 domain. Currently there are no other JH2 inhibitor in the clinical trials.

There are also several other known ligands for JH2 domain, but they all target JAK2 JH2 or TYK2 JH2. Some of them are included in Table 5. There are currently no known ligands that bind to JAK1 or JAK3 JH2 domain.

Table 5. *Ligands that bind to JH2-domain*

Protein domain	***PDB entry***	***Name***	***Citation***
JAK2 JH2	6OBB	*JAK170*	(Liosi et al., 2020)
JAK2 JH2	6OAV	*JAK146*	(Liosi et al., 2020)
JAK2 JH2	6OBL	*JAK168*	(Liosi et al., 2020)
TYK2 JH2	5TKD	-	(Moslin et al., 2017)
TYK2 JH2	6NSL	-	(Moslin et al., 2017)
TYK2 JH2	6NZR	-	(Wrobleski et al., 2019b)
TYK2 JH2	6NZQ	-	(Wrobleski et al., 2019b)
TYK2 JH2	6NZP	-	(Wrobleski et al., 2019b)
JAK2 JH2	5UT5	*GLPG0634*	(Newton et al., 2017)
JAK2 JH2	5UT4	*NVP-BSK805*	(Newton et al., 2017)
JAK2 JH2	5UT6	*diaminopyrimidine*	(Newton et al., 2017)

As can be seen from Table 5, there doesn't seem to be any JAK1 or JAK3 JH2 specific ligands in Protein Data Bank (PDB). This could in part be also due to the fact that according to Uniprot, JAK3 JH2 domain has not been characterized at all (https://www.uniprot.org/uniprot/P52333 retrieved 12.1.22) and JAK1 JH2 domain similarly only has been characterized twice (https://www.uniprot.org/uniprot/P23458 retrieved 12.1.22). The JH2 domain for JAK2 (https://www.uniprot.org/uniprot/O60674 retrieved 12.1.22) and TYK2 (https://www.uniprot.org/uniprot/P29597 retrieved 12.1.22) have been characterized multiple times, meaning that JAK1 and JAK3 JH2 domains seem to be difficult to characterize structurally.

Regardless, the lack of clinical JH2 targeting drugs means that there is unused potential that has not been rigorously pursued. Since the only other clinical JH2 binder in clinical trials targets TYK2, JAK1 and JAK3 JH2 binders would be novel in the clinical trials.

2.5 Drug screening

Drug screening is a process where new drug compounds are identified. Through scientific advances the methods of drug screening have advanced from the past. Currently the corner stones of drug screening are virtual screening and other forms of high throughput screening (HTS). These developments are important due to the high cost of drug discovery (Skardal et al., 2016).

Virtual screening is done *in silico* computationally (Śledź & Caflisch, 2018). It has now become a well-established method in drug discovery which helps screen massive amounts of compounds (Kumar et al., 2015). There are two main methods for virtual screening: ligand based and structure based (Śledź & Caflisch, 2018), as well as a method that combines the two (Kumar et al., 2015). The main difference between the two is that ligand based method starts from a known ligand whereas structure based method aims to dock potential compounds into the protein structure (Kumar et al., 2015).

The virtual screening hits need to be experimentally validated. This can be done in many forms of high throughput (HT) assays that have been developed for the purpose (Hall et al., 2016). These assays can be divided into two equally used groups: biochemical and cellular assays (Busby et al., 2020). Biochemical assays often deploy 384-well plates or even ultra-high-throughput 1536-well plates (Hall et al., 2016). Cellular assays can include for example high-throughput flow cytometry (HT-FC) or automated microscopes (Busby et al., 2020).

Virtual screening does however have challenges: majority of studies examining virtual screening hits *in vitro* has found poor correlation between *in silico* binding and *in vitro* binding (Kumar et al., 2015). Virtual screening hit should for this reason be taken with caution before experimental verification has been done.

2.6 Used methods

In this chapter, the used methods are detailed. In this thesis, the main method being used is Fluorescence polarization assay which is being used to measure half maximal inhibitory concentration (IC_{50}). LANCE® *Ultra* kinase assay is being used to study kinase

activity and flow cytometry is being used for measuring cytokine inhibition in later stage of the thesis. Size exclusion chromatography is being used for purifying the proteins that are used through the thesis.

2.6.1 Protein purification through Ni-NTA and Size-exclusion chromatography

Protein purification in this thesis happens in two steps: first one is nickel affinity and the second is size-exclusion chromatography. Two methods are used to increase the degree of purification.

Nickel affinity is an affinity purification method where nickel agarose beads are used. They have affinity towards six histidine residues which are added to the end of a recombinant protein. (Crowe et al., 1994)

Size-exclusion chromatography (SEC) is a method that is often used in protein purification (Burgess, 2018). SEC fractions molecules based on the molecular size (Ó'Fágáin et al., 2011). How it works is quite simple: the sample is run through is bead-filled column (Burgess, 2018). The beads are porous which causes the smaller molecules to get into the small pores and thus migrate at a slower rate (Ó'Fágáin et al., 2011). The sample coming out of the column can be fractioned into smaller fractions and the right fractions containing the target protein can be chosen.

2.6.2 Bradford

Bradford is a method used to measure protein concentration. In Bradford, the dye Coomassie blue G-250 binds proteins (Compton & Jones, 1985). The binding turns the dye from cationic form to anionic form which in turn changes the λ max from 470 nm to 595 nm (Kruger, 1994). This allows the quantity of the protein to be measured by measuring the absorbance in 595 nm (Compton & Jones, 1985). To determine the protein quantity from the absorbance, protein standard is established using different known amounts of proteins to calibrate the assay (Sapan et al., 1999). The standard should be linear and the used amounts should be representative of the sample to gain the needed sensitivity (Sapan et al., 1999).

2.6.3 Fluorescence polarization

Fluorescence polarization (FP) is a method that is really useful in high throughput drug screening (Hall et al., 2016). It is really effective in measuring binding (Hendrickson et al., 2020), which makes it ideal to use in research where binding is studied.

Fluorescence polarization works by having a fluorescent tracer in solution (Hall et al., 2016). This tracer has faster motion in the liquid when it is unbound and thus emits depolarized light (Hendrickson et al., 2020). When the tracer is bound to a molecule with larger molecular weight, the light emission becomes polarized, the level of which can be measured (Hendrickson et al., 2020). If a compound that can competitively bind the same

protein as the tracer is present, the level of polarization will degrease relative to the binding affinity of the competitive compound.

2.6.4 LANCE® *Ultra* kinase assay

LANCE® *Ultra* kinase assay is an assay made by Perkin Elmer. The theory behind the assay is described on the Perkin Elmer's website. Lance Ultra assay is used to assess kinase activity. It uses U*Light*™-labelled peptide and Europium conjugated anti-phospho antibody to form the assay.

The basic principle behind the assay is depicted in Figure 4. In the beginning of the assay, the kinase being studied phosphorylates one amino acid of the U*Light*-conjugated peptide. When Europium linked anti-phospho antibody binds to the phosphorylated site, europium's fluorophore is excited. Europium's fluorophore then donates the energy to the U*Light* acceptor dye. Emission is then released in 665 nm and it can be measured to determine the level of kinase activity. When kinase activity is inhibited, the level of emission is lower. (https://www.perkinelmer.com/lab-products-and-services/application-support-knowledgebase/lance/lance-tr-fret-kinase.html retrieved 1.11.22)

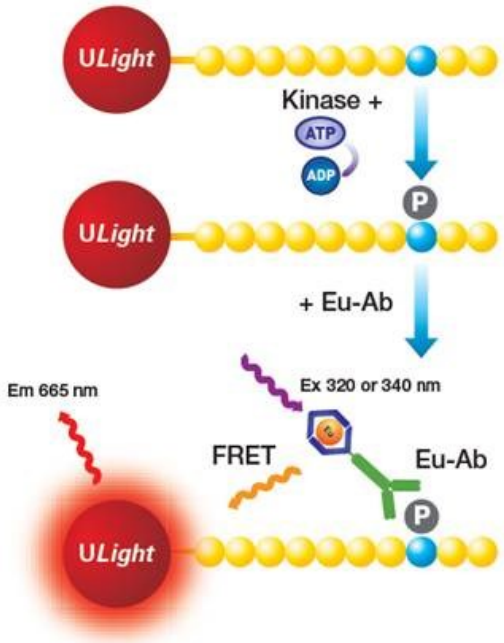

Figure 4. *The theory behind Lance Ultra assay*

2.6.5 Flow cytometry, phosphoflow and barcoding

Flow cytometry is a method that uses a light source to illuminate cells or other particles as they flow individually in a narrow stream of liquid (Givan, 2011). The light is used to acquire data on the properties of the cells (Ibrahim & van den Engh, 2007). Flow cytometry can be divided into three part: fluidics, optics and electronic (Givan, 2011).

In flow cytometry the cells need to be in suspension in order to form a stream of fluid. A wide, rapid stream called the sheath stream is used to form the narrow stream for cells. (Ibrahim & van den Engh, 2007) Cells travel in the centre of the sheath stream,

and this allows the formation of a stream that is narrow. This allows cells to be individual but does not get blocked like a normal tube would. (Givan, 2011)

Cells and their properties are detected using a laser at an analysis point (Givan, 2011). To make sure that different protein markers are registered, fluorescent dyes conjugated to antibodies are used (Ibrahim & van den Engh, 2007). The fluorochrome in the dye absorbs the illuminated light and the flow cytometer detects the emitted fluorescence. At the analysis point, the signals caused by the laser are collected by two objective lenses: forward lens and orthogonal lens. (Givan, 2011) Forward scattered light (FSC) is light that is detected by the forward lens and is scattered in proportion to the size of the particle. The orthogonal lens detects side scattered light (SSC) is scattered perpendicular in proportion to the intracellular granularity. This information can be combined and used to differentiate the cells based on morphology. (Ibrahim & van den Engh, 2007) Combined with the information on morphology and fluorescent dyes, it is possible to gather a lot of information on different kinds of cells.

The final stage of flow cytometry is electrical, where the signals collected by the detectors is converted into voltage and further into data that is processed on a computer (Givan, 2011). The data can be further processed to acquire information on the studied cells (Givan, 2011).

Phosphoflow, also known as phosflow is a technique used to study immune response by measuring the phosphorylation of intracellular signalling molecules in leukocytes (Wu et al., 2010). The phosphorylation status is studied using highly specific antibodies (Spurgeon & Naseem, 2020) that are conjugated with small fluorophores (Wu et al., 2010). Phosflow is really useful because it makes it possible to differentiate cell subpopulations which can then be analysed simultaneously (Wu et al., 2010).

While phosflow is already good for high-throughput drug screening, the screening capacity can be further increased by using fluorescent cell barcoding to stain the samples (Wu et al., 2010). It allows up to 96 samples to be run in the same tube saving a lot of time and reagents while also helping to reduce variation between samples (Spurgeon & Naseem, 2020). Fluorescent barcoding is done by labelling cells with different concentrations of two fluorophores before antibody staining (Wu et al., 2010). Samples can then be separated by the fluorophore concentration and analysed as if they were independently acquired (Spurgeon & Naseem, 2020).

3 AIMS OF THE STUDY

The aim of this study is to screen potential JAK1 and/or JAK3 inhibitors that have specificity towards the JH2 domain of JAK1 or JAK3. These inhibitors should inhibit the JH2 domain from activating the JH1 domain thus inhibiting the function of the whole protein. This specificity would allow better JAK specificity and ideally this way minimize harmful side effects in potential clinical use. The main goal of this study is to use these compounds for the purposes of immune disorder treatment. The secondary goal of this study is to find inhibitors that would inhibit hyperactive JAK mutation found in hematopoietic malignancies, leukaemia, lymphomas, and cancer. JH2 selective binders found in this thesis could also be used as probe-compounds in research.

4 MATERIALS AND METHODS

4.1 Protein production and purification

New batch of proteins was produced for the experiments that followed set 1. Protein expression was done using Bac-to-Bac® Baculovirus Expression System by Invitrogen. Bacmids were extracted from previously cultured DH10Bac bacteria stored in 50% glycerol in -80´C. Defrosted and inoculated DH10Bac were grown in 5 ml of Lysogeny broth with 50 μg/ml kanamycin, 7 μg/ml gentamycin and 10 μg/ml tetracycline overnight at 37°C on a shaker.

The extraction of the bacmid was done first pelleting 2 ml of the cell suspension by centrifuging it in 6800 g for 3 minutes at RT. The pellet was resuspended to Solution I (15 mM Tris-Hcl pH 8, 10 mM EDTA, 200 μg/ml RNase A RNase A). Solution II (0,2 M NaOH, 1% SDS) was added and the sample was incubated in RT for 5 minutes. Solution III (3 M potassium acetate pH 5,5) was added, and the sample was incubated on ice for 10 minutes. The sample was centrifuged in 16100 g for 20 minutes at 4°C. The supernatant was transferred to 2-propanol and incubated on ice for 5 minutes. The sample was centrifuged in 16 100 g for 15 minutes at RT. The precipitate was washed twice with 70% ethanol and centrifuged for 5 minutes at RT. The rest of the ethanol was allowed to evaporate in sterile cell culturing hood and the dry sample was eluted to 40 μl of sterile TE overnight at RT.

Expression was done in Sf9 and Hi-5 cells by laboratory technicians due to limitations of antibiotic free laboratory access.

Table 6. *Cells and bacmid virus percentages used to produce protein*

Protein construct	***Boundaries***	***Used cells***	***Virus %***
JAK1 JH1 D1003N	864-1155-His	Hi-5	5%
JAK1 JH2	553-836-His	Hi-5	5%
JAK2 JH1	836-1132-His	Hi-5	10%
JAK2 JH2	503-827-His	Hi-5	5%
JAK3 JH1	811-1124-His	Sf9	5%
TYK2 JH2	564-1188-His	Hi-5	10%

Purification was done by freezing and thawing the cells three times. After the first freeze, 2 times the volume of the cells' worth of HIS Lysis Buffer (20 mM Tris-HCl, 500 mM NaCl, 10% glycerol, 20 mM Imidazole 1 mM sodium vandate, 1mM PMSF, 1 μg/ml Pepstatin A, 1 μg/ml Leupeptin, ≥ 5 units/ml benzonase and 5 mM $MgCl_2$) was added. 2 μl fraction of cell lysate was taken for SDS-PAGE (fraction 1). Second fraction was prepared by taking a separate sample of cell lysate and centrifuging it in 16 100 g for 20 minutes at 4°C. The pellet was suspended in 500 μl of lysis buffer and a 2 μl fraction was taken (fraction 2). The protein sample was centrifuged in 10 000 g for 30 minutes at 4°C. Supernatant was collected and added to a tube with washed Ni-NTA beads. The sample

was rotated for 1 hour at 4°C. The sample was centrifuged in 2147 g for 8 minutes at 4°C. The pellet was washed 3 times in His Wash Buffer (20 mM Tris-HCl, 500 mM NaCl, 10% glycerol and 30 mM Imidazole) by centrifuging in 2147 g for 5 minutes at 4°C. Elution was done by adding 1.2 ml and 1.1. ml of elution buffer (Lysis Buffer without inhibitors, containing 0.25 M Imidazole). The sample was centrifuged in between first and second elution collection in 2147 g for 5 minutes at 4°C. The purity of the sample was assessed by performing SDS-PAGE to fraction 1, fraction 2 and a small sample of elution.

The eluate was run through Sephadex™ G-25 M PD-10 desalting column made by GE healthcare to exchange the buffer to PD-10 buffer (20 mM Tris-HCl, 300 mM NaCl, 10% glycerol and 0.5 mM TCEP). The samples were concentrated using 10 kMw-concentrator-tubes by MERCK Millipore by centrifuging in 4000 g for couple of minutes at a time in 4°C until the samples were 2 ml in volume.

The sample was then filtrated through Size-exclusion Chromatography (SEC) column HiLoad® 16/600 Superdex® 200 pg made by GE healthcare. Fraction containing target protein were collected and concentrated prior to protein concentration determination..

The protein concentration was determined using Thermo Scientific NanoDrop 2000. If the absorbance was below 0.1, Bradford was done to determine the protein concentration of the sample. Bradford was done for undiluted, 2x-dilution, 5x-dilution and 10x dilution of the studied protein stock. The protein concentration was determined by comparing a suitable absorbance to a protein standard consisting of 2x serial dilution in 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml, 0.625 µg/ml, 0.3125 µg/ml, 0.15625 µg/ml, and 0.078125 µg/ml.

To find the protein concentration that produced the right signal level proteins were titrated by 2x serial dilution in 50 nM, 25 nM, 12.5 nM, 6.25 nM, 3.125 nM, 1.5625 nM, 0.78125 nM, 0.390625 nM and 0.1953125 nM to find Bmax. Bmax was calculated in Graphpad Prism 9.0.0 using nonlinear regression (curve fit) one site-specific binding analysis. 70% of Bmax was chosen and corresponding molarity was extrapolated in Graphpad.

4.2 Compound sets

Table 7. *Different sets of compounds and the experiments done before this thesis*

Set	*Target JAK and domain*	*Screen*	*Number of hits*	*Hit verification*	*JAK-selectivity profiling*	*JAK activity inhibition*	*Cytokine signalling inhibition*
1	JAK3 JH2	Virtual binding	156	Not done	Not done	Not done	Not done
2	JAK1 JH2	Binding	25	Done	Not done	Not done	Not done
3	JAK3 JH2	Binding	6	Done	Done	Done	Not done

The compounds used in this thesis are detailed in Table 7. Set 1 composed of new hits identified *in silico* by Atomwise and the experiments started from the beginning with confirming the binding *in vitro*. Set 2, identified originally in small-scale binding screen for a set of kinase inhibitors and ATP-analogues, had been verified experimentally before the start of this thesis. For the set 3, which was identified in a small-scale binding screen, hits had been previously verified as well as binding and JAK-activity inhibition characterization had been performed earlier. Sets 2 and 3 enter the study at different stages for these reasons.

4.2.1 Hit verification

The experimental *in vitro* hit verification was done for the virtual screening hits with Fluorescence polarization (FP) for set 1.

Protein samples were prepared into a reaction buffer that consisted of 20% glycerol, 20 mM Tris-HCl pH 8, 150 mM NaCl and 0.01% Brij-35. Just before the protein and non-protein samples were prepared, 2 mM of DTT was added to the reaction buffer. The fluorescent tracer used in the verification was Bodipy FL labelled JNJ-770662 in 1.5 nM concentration. The molarity of the different JAK proteins in the samples are depicted in Table 8. The concentration was titrated in similar manner to section 4.1 before the start of this thesis.

Table 8. *Molarity of the protein samples used in the hit verification process*

Protein	***Molarity (nM)***
JAK1 JH2	1.3
JAK2 JH2	52
JAK3 JH2	1000
TYK2 JH2	24

In the hit verification set 1 of compounds were used. These 156 compounds were sent by company called Atomwise who had screened the binding computationally. 2.5 µl of compound mixture was added to black 384-plate (ProxiPlate-384 F Plus, PerkinElmer) in two replicas. JNJ-7706621 was used as a positive control and DMSO as a negative control, both of which were diluted into reaction buffer without DTT 50x. On top of the compound mixture, 2.5 µl of protein mixture was added to each well. The plate was centrifuged in 400 g for 1 min. Finally, the plate was imaged using PerkinElmer Envision plate reader with Fluorescence polarization program with 480 nm excitation, 535 nm emission filters.

The compounds were delivered in two plates. Compounds of the first plate were tested in 10 µM and 1 µM for the JH2 domains of all JAK proteins in two replicas. Furthermore, the compounds were tested at 100 µM and 10 µM concentration for JAK3 to detect putative low-affinity binding. The compounds of the second plate were tested in the same manner, but in 50 µM and 10 µM to avoid possible precipitation in 100 µM concentration. The second plate was also tested for all the JH2 domains of JAKs.

Samples were chosen for verification if the signal lowered in a concentration dependent manner. Verification was done using the same assay, but as a 2x serial dilution in 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM, 3.125 µM and 1.5625 µM in three replicas. JNJ-7706621 and DMSO were used as controls as before. The results were processed in Graphpad Prism 9.0.0 using nonlinear regression (curve fit) [inhibitor] vs. response (three parameter) analysis to obtain IC_{50} values. The top and bottom value of JNJ inhibition curve were used as top and bottom constraints respectively to ensure that the linear regression was done for the whole curve and not just parts of it.

4.2.2 JAK selectivity profiling

JAK selectivity profiling was done for compounds from set 2 and hits from set 1. The binding selectivity between JH2 and JH1 domains and between different JAKs were assessed.

Compounds were tested using FP in 10x dilution series (100 µM, 10 µM, 1 µM, 100 nM, 10 nM, 1 nM, 0.1 nM) with 3 protein replicas and 1 non-protein replica. Fluorescence polarization was done as described in Section 4.2.1.

The compounds were tested for JAK1 JH2, JAK2 JH2, JAK3 JH2 and TYK2 JH2 as well as for JAK1 JH1, JAK2 JH1, JAK3 JH1 and TYK2 JH1. All the JH2 domains expect for JAK3 JH2 used were a new lot of protein, so new molarity was determined by titration (described previously in Section 4.1). The molarity of JH2 and JH1 domains used ate described in Table 9. IC_{50} values were calculated as described in Section 4.2.1.

Table 9. *Molarity of the different JAK domains used in the experiments*

Protein	***Domain***	***Molarity (nM)***	***Domain***	***Molarity (nM)***
JAK1	JH2	14	JH1 DN	26
JAK2	JH2	76	JH1	34
JAK3	JH2	1 000	JH1	46
TYK2	JH2	66	JH1	74

4.2.3 Lance ultra, JAK activity inhibition

Lance ultra was done for hits from set 1 and set 2, with some compound being excluded from set 2. To determine the protein concentrations of JAK JH1-2 stocks, Bradford was performed.

LANCE® Ultra kinase assay by Perkin Elmer was done using ProxyPlate™ as per manufacturer's protocol. Kinase buffer used contained 50 mM Tris-HCl, 10 mM $MgCl_2$, 0.5 mM TCEP, 0.01% Brij, 1 mM EGTA and 0.05% BSA. The compounds were tested for JAK1 JH1-2 and JAK3 JH-2 in molarities presented in Table 10. The plates were measured using PerkinElmer Envision plate reader and a time-resolved fluorescence program with 320 nm excitation and 665 nm emission filters.

Table 10. *Molarity of different JAK domains used in the experiments*

Protein	***Molarity (nM)***
JAK1 JH1-2	133.667
JAK3 JH1-2	4.025

Before the data was analysed, background signal was removed. The IC_{50} values were calculated as described in Section 4.2.1.

4.2.4 Phosflow, cytokine signalling

Phosflow was done for the compounds that had been verified to bind JAK JH2 domain. Phosflow was done using peripheral blood mononuclear cells (PBMC) extracted from the blood of three healthy volunteering donors. The cells were extracted from whole blood using ficoll gradient by a lab technician and frozen for later use. Each of the compound was tested in three different concentrations: 10 µM, 0.5 µM and 0.025 µM. Baricitinib was used as a positive control. Uninhibited cells both stimulated and non-stimulated were used as a control. The inhibition was done in +37°C for 60 minutes.

For each donor the compound were stimulated with IL-6 and either IL2 or IL4 in 100 ng/ml and pSTAT5 and pSTAT3 levels were studied for the interleukins respectively. Due to issues with IL-2 stimulation detection for the last donor cells IL-4 with pSTAT6 was used instead. Lyse/fix buffer by BD Biosciences was used to stop the stimulation after 15-minute incubation in +37°C. Methanol permeabilization was done by incubating the samples on ice for 10 minutes and by keeping the samples in -80°C overnight.

Antibodies used in the experiment were: 2µl of PEcy7-linked anti-CD33 (BD) [Cat 333952], 25 µl of PerCP-linked anti-CD3 (BD) [Cat 345766] in and 2 µl of APCcy7-linked anti-CD4 (Invitrogen) [Cat 47-0047-42] To study the pSTAT levels, 20 µl of PE-linked anti-pSTAT antibodies (BD) were used. pSTAT3 (pY705) [Cat 812567] pSTAT5 (pY694) [Cat 612567] and pSTAT6 (pY641) [Cat 612701] were used to study the stimulation of IL6, IL2 and IL4 respectively.

Barcoding was done by using -NHS esters pacific blue and pacific orange in concentrations of 0 µg/ml, 0.15 µg/ml, 0.45 µg/ml, 1.35 µg/ml, 3 µg/ml and 7,5 µg/ml. Due to issues with separating the barcoded populations in data-analysis of the first two experiments, the last experiment was carried out using concentrations of 0µg/ml, 0.15 µg/ml, 0.53 µg/ml, 1.84 µg/ml, 6.43 µg/ml and 22,51 µg/ml.

The flowcytometry was carried out using CytoFLEX S flow cytometer (Beckman Coulter). The data was analysed using FlowJo™ by Becton, Dickinson & Company (BD) and Flowing Software which was made by Perttu Terho from Turku Centre for Biotechnology. In FlowJo analysis, lymphocytes were studied. Barcoded samples were gated by the barcoding to identify original subpopulations. This data was imported to Flowing Software and pSTAT levels were assessed for each barcoded lymphocyte subpopulation.

The pSTAT data was normalized from 0 to 1 relative to the non-stimulated non-inhibited control and stimulated non-inhibited control. Datapoints that were higher than 1.5 or had less than 50 events were excluded. The IC_{50} values were calculates using

GraphPad nonlinear regression (curve fit) [inhibitor] vs. response (three parameter) analysis. Slopes that had R^2 value less than 0.3 were excluded.

5 RESULTS AND DISCUSSION

5.1 Virtual screening

Two 96-plates of compounds found by virtual screening were screened for binding to JAK JH2 domains. There were 156 compounds in total that were screened. In the first plate, some preliminary hits were found, but further verification showed no binding. For the second plate, some preliminary hits were found for JAK1 and JAK3 (see Table 11). No preliminary hits were found for JAK2, and one hits was found for TYK2 (not detailed here).

Table 11. *Preliminary hits and the results from verification*

Compound	***Preliminary binding JAK1 JH2***	***Preliminary binding JAK3 JH2***	***Verified binding JAK1***	***Verified binding JAK3***
1F	x		Weak binding	No binding
1H		x	No binding	No binding
3B	x		No binding	No binding
3H	x		No binding	No binding
3C	x		Weak binding	No binding
3D	x		No binding	No binding
4B	x		Binding	Binding
4G	x		Binding	No binding
5D	x	x	No binding	No binding
6D		x	No binding	Weak binding
8D	x	x	Binding	Binding
10B	x		Weak binding	No binding
10C	x		Weak binding	No binding

Table 11 shows the results of the verification for the preliminary hits. Three compounds were found to bind either JAK1 JH2 or both JAK1 JH2 and JAK3 JH2. These compounds were 4B, 4G and 8D. Compounds 5D and 3D highly fluorescent, which might result with decrease in fluorescence polarization. The compounds were thus considered as putative false positives and excluded from further analyses. If the IC_{50} value that was calculated on GraphPad was over 100 (µ-molar) it was excluded as a weak binder. Table 12 shows average IC_{50} values and standard deviation of the final hits for all JAK JH2 domains.

Table 12. *The average IC_{50} values (μM) and the SD of the compounds for JH2 domains*

	JAK1 JH2	***JAK2 JH2***	***JAK3 JH2***	***TYK2 JH2***
4B	28.2 ± 4.3	ND	62.8 ± 5.1	ND
4G	49.1 ± 9.3	ND	ND	ND
8D	76 ± 14.2	ND	41.2 ± 3.7	ND

The hits found showed binding that was below the set 100 μM IC_{50}. The binding was still on the weaker side as it was not nanomolar. The low number of hits could be explained by the fact that all the known JAK inhibitors were removed from the virtual screening set by Atomwise. This resulted in only weak binders being left into the final compound set. The found compounds were however novel binders.

5.2 JAK selectivity profiling

JAK-selectivity was assessed by testing the binding for JH1 and JH2 domains for each JAK. IC_{50} values were calculated from the data and an example of a classical binding slope can be seen in Figure 5.

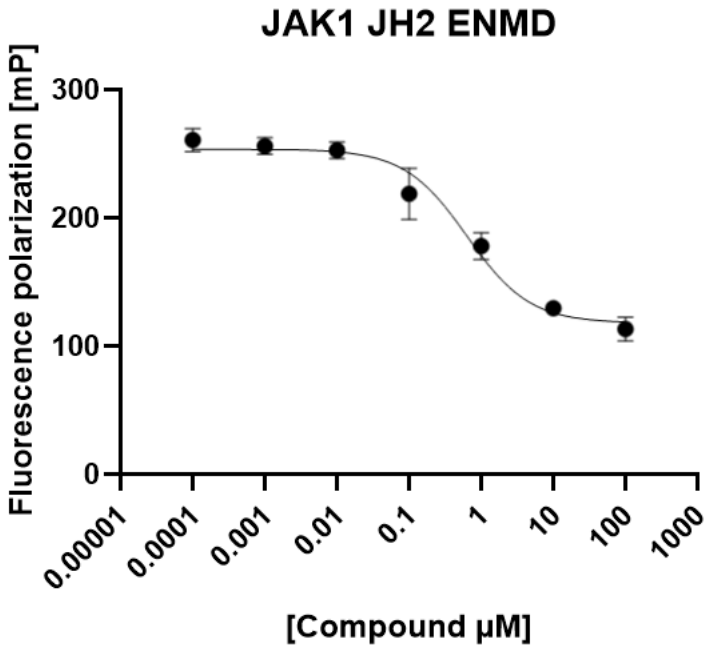

Figure 5. *Dose-response binding curve for compound ENMD*

Table 13 shows that binding is generally best for JAK1 although some binding is observed also for TYK2. As expected, almost all the compounds showed JAK1 JH2 binding, which is not surprising since the compound set was screened for JAK1 JH2 domain. Good submicromolar binders for JAK1 JH2 domain were AZD5 with an IC_{50}-value of 0.96

μM and AT with and IC_{50}-value of 0.21 μM. For TYK2 JH2 domain sub micromolar binders included CYC116 with an IC_{50}-value of 0.29 μM, ENMD with an IC_{50}-value of 0.18 μM, F165 with an IC_{50}-value of 0.19 and finally AT with an IC_{50}-value of 0.08 μM.

Table 13. *The average IC_{50} values (μM) and standard deviation for each JH2 domain*

Compound	***JAK1***	***JAK2***	***JAK3***	***TYK2***
Mito	31 ± 15	ND	ND	17 ± 8.1
AZ960	8.5 ± 2.8	ND	ND	ND
Momelo	70 ± 24	ND	220 ± 130	2.9 ± 2.4
CYC116	4.1 ± 2.3	ND	ND	0.29 ± 0.3
Picti	10 ± 5.1	ND	ND	ND
AZD5	0.96 ± 0.33	110 ± 19	57 ± 28	9.2 ± 0.1
TG100	4.1 ± 1.4	20 ± 3.2	18 ± 2.2	11 ± 4.1
Omipa	36 ± 2.1	ND	ND	ND
TG101	10 ± 2.8	ND	91 ± 28	9.1 ± 1.9
TAK901	11 ± 3.4	ND	ND	8.3 ± 5.5
KW	2.2 ± 0.26	220 ± 110	120 ± 52	26 ± 8.7
Crizo	26 ± 3.8	ND	ND	19 ± 17
ENMD	1.8 ± 0.31	33 ± 14	57 ± 24	0.18 ± 0.07
VX680	50 ± 36	25 ± 20	ND	230 ± 150
AG14	ND	ND	ND	300 ± 140
TAE684	15 ± 3.4	ND	ND	39 ± 23
AG12	150 ± 13	ND	ND	ND
F172	6.1 ± 2.8	ND	ND	4.3 ± 3
F7	8.6 ± 4.5	68 ± 25	160 ± 210	35 ± 12
F165	8.3 ± 3.1	ND	ND	0.19 ± 0.085
F143	13 ± 1.8	370 ± 220	89 ± 9.8	11 ± 8.2
AT	0.21 ± 0.089	5.9 ± 2.3	6.1 ± 1.1	0.08 ± 0.018
F13	8.6 ± 1.6	240 ± 47	130 ± 85	13 ± 3.3
F135	12 ± 2	120 ± 26	48 ± 3.5	1.9 ± 0.51

Compounds with IC_{50} value 500 or higher were deemed to not be binders. ND is used to signify that binding was not detected.

When the JH2 data on Table 13 is compared with the JH1 data seen on Table 14, it can be seen that many compounds have higher binding affinity towards JH1 domain. This can be better seen in Figures 6 through 9 where the fold value for JH2 binding relative to JH1 binding has been calculated.

Table 14. *The average IC_{50} values (μM) and standard deviation for each JH1 domain*

	JAK1	***JAK2***	***JAK3***	***TYK2***
Mito	>500	>500	>500	>500
AZ960	0.02 ± 0.004	0.01 ± 0.001	0.01 ± 0.005	0.01 ± 0.001
Momelo	0.1 ± 0.03	0.01 ± 0.002	0.04 ± 0.01	0.01 ± 0.002
CYC116	3.7 ± 1.77	0.06 ± 0.02	0.4 ± 0.16	0.7 ± 0.91
Picti	>500	270 ± 76	125 ± 46.16	>500
AZD5	5.3 ± 1.5	2.4 ± 0.9	1.8 ± 0.68	6.1 ± 1.9
TG100	172 ± 38	23 ± 1.1	62 ± 9.6	68 ± 9.7
Omipa	>500	316 ± 63	323 ± 76	>500
4B	221 ± 24	8.7 ± 2.3	39 ± 8.6	25 ± 5.7
TG101	0.42 ± 0.24	0.03 ± 0.02	0.1 ± 0.06	0.08 ± 0.08
TAK901	0.68 ± 0.46	0.06 ± 0.04	0.02 ± 0.01	0.1 ± 0.02
KW	1.06 ± 0.9	0.3 ± 0.03	0.2 ± 0.08	0.2 ± 0.03
Crizo	0.36 ± 0.07	0.06 ± 0.03	0.4 ± 0.18	0.3 ± 0.31
ENMD	3.1 ± 1.4	0.1 ± 0.07	0.2 ± 0.03	0.1 ± 0.03
VX680	9.4 ± 2.7	0.7 ± 0.27	3.8 ± 0.11	2.9 ± 1.96
AG14	235 ± 154	54 ± 22	365 ± 181	>500
TAE684	1.5 ± 1.1	1.1 ± 0.19	0.4 ± 0.16	1 ± 0.27
8D	78 ± 21	22 ± 2.2	53 ± 15	16 ± 5.8
AG12	>500	>500	>500	>500
F172	116 ± 43	169 ± 92	103 ± 3.7	>500
F7	>500	302 ± 55	443 ± 124	>500
F165	>500	>500	>500	>500
F143	87 ± 26	135 ± 17	195 ± 38	52 ± 27
AT	0.04 ± 0.01	0.01 ± 0.004	0.01 ± 0.01	0.01 ± 0.001
F13	61 ± 49	31 ± 3.8	23 ± 16	>500
F135	35 ± 13	43 ± 7.1	55 ± 24	85 ± 49
4G	>500	>500	>500	>500

Compounds with IC_{50} value 500 or higher were deemed to not be binders. ND is used to signify that binding was not detected.

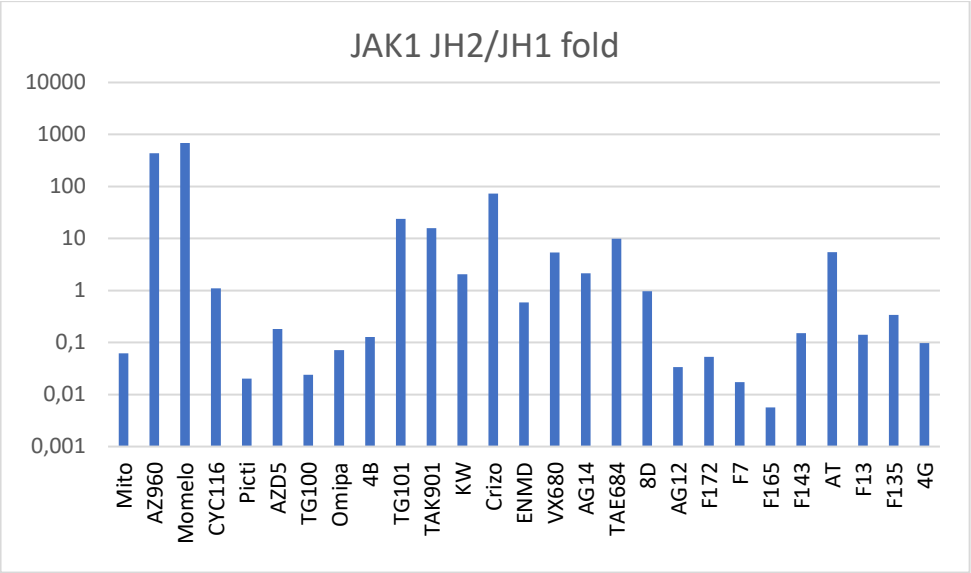

Figure 6. *IC_{50} values (μM) for JAK1 by JH2 and JH1 domains. Compounds for which binding was not defined have had a calculator value of 500 μM*

The data in Figure 6 shows that there are many potentially JH2 selective binders for JAK1 that bind JH2 domain but that don't bind JH1 domain very well. These include Mito, Picti, TG100, Omipa, 4B, F172, F7, F165, F143 and 4G. For example, F13 and F135 look promising in terms of JH2 binding, but it can be seen from the Table 14 that the IC_{50}-value for JH1 binding is also very low, meaning that these do not bind specifically to JH2. The number of strong JH1 binders is a high, but it is expected that in binder screenings large portion of the compounds are deemed unsuitable at one point or another.

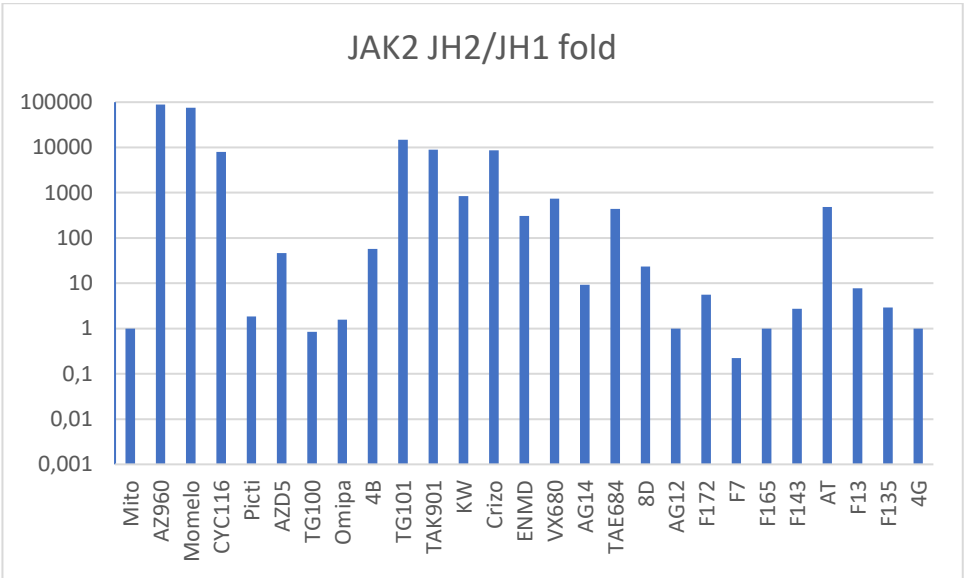

Figure 7. *Fold values of JH2 binding relative to JH1 binding for JAK2. Compounds for which binding was not defined have had a calculator value of 500 μM*

As expected, Figure 7 shows that generally the compounds show little binding affinity towards JAK2 JH2 domains. This is a good thing, as it means that the compounds are specific towards JAK1 over JAK2. There is however strong JAK2 JH1 binding over JH2 binding that can be seen for example in AZ960 and KW. Of the potential compounds

identified in the experiments with JAK1, TG100 exhibits quite strong JH2 and JH1 binding for JAK2. F7 exhibits some JH2 binding for JAK2 with an IC_{50} value of 68 µM, but there is a possibility that the binding is not strong enough to have clinical relevance. Other compounds like F172 do also show binding as well, but the IC_{50}-value is 100 µM or above, which means that they are definitely weak binders. This shows that the compounds are different in terms of JAK selectivity.

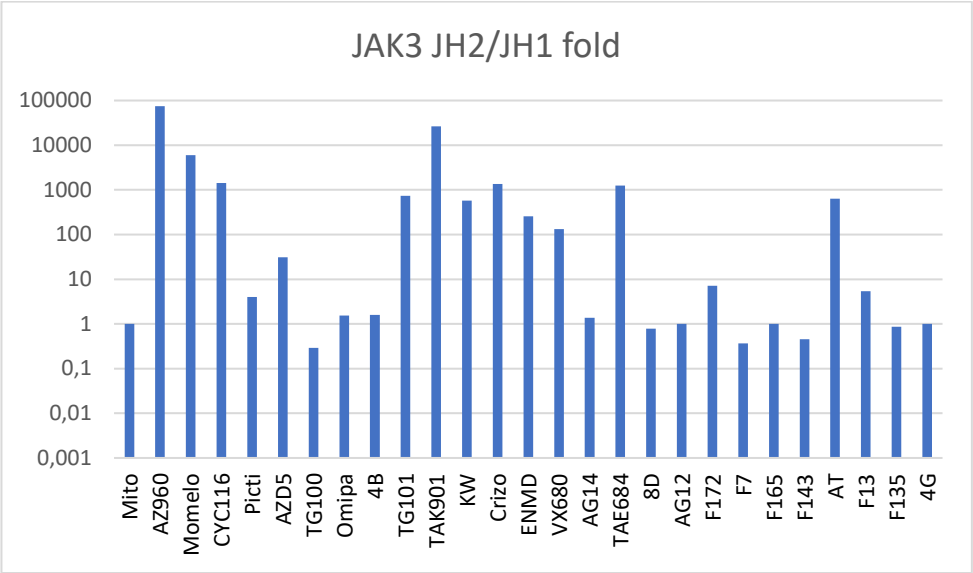

Figure 8. *Fold values of JH2 binding relative to JH1 binding for JAK3. Compounds for which binding was not defined have had a calculator value of 500 µM*

As can be seen from Figure 8, there doesn't seem to be any JH2 specific binders for JAK3. This isn't too surprising considering that the set 2 was for JAK1 and the three compounds from the JAK3 specific set (4B, 4G and 8D) don't seem JH2 specific for JAK3 even if 4B and 8D do show binding affinity. From the JAK1 candidates, TG100 and 4B show binding to both JH2 and JH1 domains for JAK3, which makes them not exclusively specific to JAK1. F143 shows also some JH2 binding for JAK3, but the IC_{50}-value is almost 100 µM making it a rather weak binder.

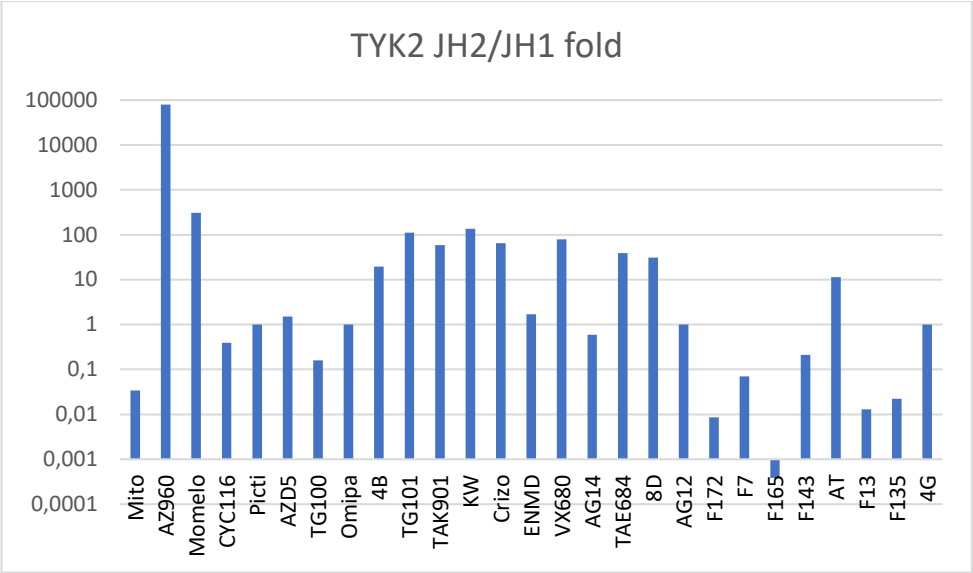

Figure 9. *Fold values of JH2 binding relative to JH1 binding for TYK2. Compounds for which binding was not defined have had a calculator value of 500 µM*

Figure 9 shows that there are larger number of strong binders for TYK2 than it could be expected. From the JAK1 candidates Mito, F172, F7 and F165 show binding to TYK2 JH2 domain. Furthermore, TG100 and F143 show binding to both JH1 and JH2 domain of TYK2. 4B binds to TYK2 JH1 domain but either 4B or 4G do not bind to TYK2 JH2 domain.

Overall, it seems that compounds specific to JAK1 JH2 domain are Picti, Omipa and 4G. The binding slopes of Picti for JAK1 JH2 and JH1 are shown in Figure 10. Binders exclusive to JAK1 and TYK2 JH2 domain are Mito, F172, F7 and F165. F7 shows an IC_{50}-value of 68 µM towards JAK2 JH2 domain and F143 and IC_{50}-value of 89 µM towards JAK3 JH2 domain, so the binding was on the weaker side with both compounds.

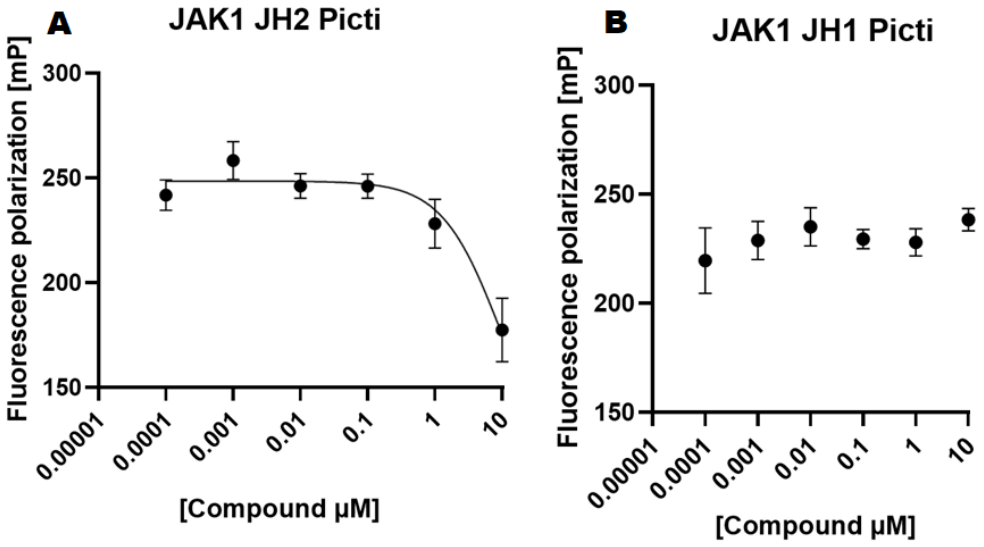

Figure 10. *A) Compound Picti, fluorescence polarization as a function of concentration for JAK1 JH2, B) Compound Picti, fluorescence polarization as a function of concentration for JAK1 JH1*

Figure 10 shows an example of a binding slope for a compound that had JH2 selective binding for JAK1. The figure A shows the binding slope, but figure B shows that no concentration dependent change in fluorescence polarization can be seen for JAK1 JH1 domain.

5.3 JAK activity inhibition

Lance Ultra assay provided results that showed that inhibition was generally widely seen across different compounds for both JAK1 and JAK3. Especially compounds that had JH1 binding showed good inhibition.

Table 15. *The average IC_{50} values (μM) and SD for JAK1 and JAK3 inhibition*

Compound	***JAK1***	***JAK3***
AZ960	0.019 ± 0.008	0.21 ± 0.08
AZD5	17 ± 10	13 ± 1
Crizo	0.57 ± 0.15	3.9 ± 1.0
CYC116	2.0 ± 1.0	1.4 ± 0.4
ENMD	1.5 ± 0.6	4.3 ± 1.4
KW	1.5 ± 0.2	4.3 ± 1.2
Mito	ND	47 ± 31
Momelo	0.14 ± 0.09	0.28 ± 0.08
Omipa	120 ± 49	360 ± 330
Picti	Possible actv.	ND
TAK901	0.51 ± 0.07	0.26 ± 0.03
TG100	190 ± 81	ND
TG101	0.03 ± 0.03	0.23 ± 0.16
VX680	60 ± 12	100 ± 22
4B	19 ± 15	31 ± 35
4G	ND	2.8 ± 4.7
8D	240 ± 94	7.0 ± 2.6
AT	0.29 ± 0.05	0.031 ± 0.053
F13	140 ± 45	3.4 ± 5.1
F143	ND	ND
F165	ND	1.7 ± 1.8
F172	160 ± 140	29 ± 37
F7	ND	ND
TAE684	13 ± 2	0.32 ± 0.38
F135	38 ± 8	0.023 ± 0.016

Compounds with IC_{50} value 500 or higher were deemed to not be binders. ND is used to signify that binding was not detected.

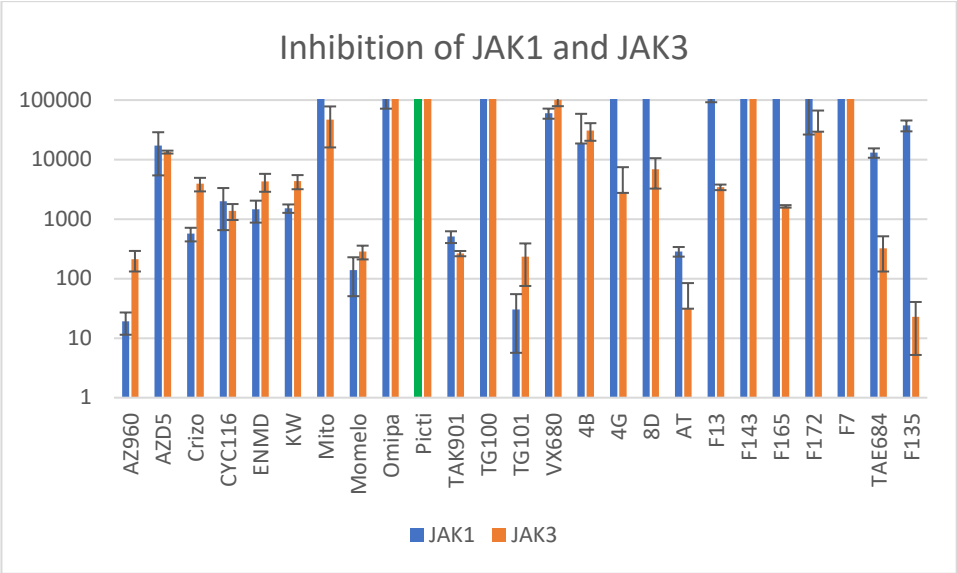

Figure 11. *IC_{50} values (nM) for inhibition rates for JAK1 and JAK3. Values over 100 000 are presented as 100 000. Green column indicates possible activation*

From Table 15 and Figure 11 it can be seen that of the exclusive JAK1 JH2 binders Picti and Omipa, both seem unsuitable as inhibitors. Picti is possibly an activator of JAK1 instead of inhibitor and the IC_{50} value of Omipa inhibition is high. Also 4G, which is likewise an exclusive JAK1 JH2 binder shows high IC_{50} value for JAK1 inhibition.

Mito, F172, F7 and F165, which showed exclusive binding to JAK1 and TYK2 JH2 domains all also don't seem good inhibitors of JAK1 based on the data. Of the 4 compounds, only F172 has IC_{50} value below 500 with a value of 160. The IC_{50} value is still over 100, although the standard deviation is also high. This means that perhaps more replications could help gain more trustworthy IC_{50} value.

Overall, the compounds that showed inhibition, showed inhibition to both JAK1 and JAK3. This is surprising, because the compounds were more suitable for JAK1 binding than JAK3 binding since most of them were screened for JAK1 specifically. This could be explained by JH1 binding. The reason for this is that all the good inhibitors for JAK1 showed binding to JH1. Some, like Momelo showed strong binding only to JH1 domain, but most, like AZ960, KW and AT showed binding to both JH1 and JH2 domain. The case is similar with JAK3. Compounds like AZ960, Crizo and KW show binding to JH1 domain while for example AT shows binding to both JH2 and JH1. The key difference between compounds that show inhibition and the compounds that don't, seems to be JH1 binding. This could mean that although the compounds bind to JH2, the binding was not enough to inhibit the activity. The same might not be true for JH1 binding, which could lead to inhibition more easily than JH2 binding. It is also possible that the mechanism behind the inhibition is different and unrelated to regulation of JH1 activation via JH2 inhibition.

Interestingly, 4G and F165 show virtually no binding to either JAK3 JH1 or JH2 domain, yet they exhibit good inhibition of JAK3. For both compounds, the standard deviation was larger than the average, so the results need to be read cautiously. Regardless, if full length JAK3 was used in the studies, the inhibition could be explained by binding to other part of the protein, but since only the JH2-JH1 domain of JAK3 were used to study the inhibition, this can't explain the results. The only thing present in JAK3

JH2-JH1 construct that was not present in either JH2 or JH1 domain is the section between the domains, with boundaries of 791-810. According to Uniprot, only JH1 domain has been crystallized and only predicted AlphaFold structure exists (https://www.uniprot.org/uniprot/P52333 retrieved 14.1.2022). Even the AlphaFold structure shows that the prediction is uncertain in that region (https://alphafold.ebi.ac.uk/entry/P52333 retrieved 14.1.2022) so it isn't possible to make any estimations on how this region could affect the protein activity based on three-dimensional structure.

5.4 Cytokine signalling inhibition

The cytokine signalling studies were done to gain better understanding on the inhibitory potential of the compounds. Lance Ultra studies JH1 activation and the compounds might inhibit the JAKs though other mechanisms. It was possible to estimate the IC_{50}-value for inhibition for some compounds and generally the results are sub micromolar. This is promising news, since it means that the compounds inhibit JAK1 or JAK3 activity in the cells.

Table 16. *The IC_{50} values (μM) based on the cytokine signalling inhibition*

Compound	***IL6***	***IL4***
Baricitinib	0.012 ± 0.015	ND
AZ960	0.1 ± 0.039	0.016
Crizo	0.027	ND
CYC116	0.33 ± 0.29	ND
ENMD	0.47	ND
F172	0.032	ND
KW	0.22	ND
Mito	0.14	ND
Momelo	0.13 ± 0.013	2.4
Omipa	10	ND
Picti	ND	5.2
TAK901	0.82	0.29
TG100	0.066	ND
TG101	0.093	ND
VX680	5	ND
4B	12 ± 9.6	ND
4G	78	ND
8D	7.9	0.31
AT	0.018	0.014
BX795	0.038 ± 0.031	0.027
BX912	0.35	ND
JNJ	ND	0.0069
F135	0.53	ND
F143	0.45	ND
F165	11	ND

Table 16 shows the µM IC_{50} values for the compounds for which IC_{50} value was possible to be determined. If the compound resulted with IC_{50} value from multiple replicas, the IC_{50} value is an average and standard deviation is also presented. This lack of replicas is because the cytokine signalling studies had issues. The barcoding was not successful, leading to issues with identifying the populations. The barcoding was chosen to improve high throughput of the studies and it had worked previously on experiments using fresh cells. Furthermore, IL2 stimulation failed twice, for which reason IL4 was chosen to the third replica. IL4 was chosen because it also signals through JAK1/JAK3 like IL2 does (George Abraham et al., 2020). This led to IL4 having only one replica. The compounds were also only tested in three different concentrations as a way to improve high throughput and to screen which compounds should be selected for further studies. For these reasons, the IC_{50} values presented here are estimates and should be taken cautiously. If the R^2 value was below 0.6 the data has been left unanalysed due to low reliability and the IC_{50} value is presented as ND. This also resulted in some compounds not having any reliable replicas and the IC_{50}-value estimates are not thus shown to them.

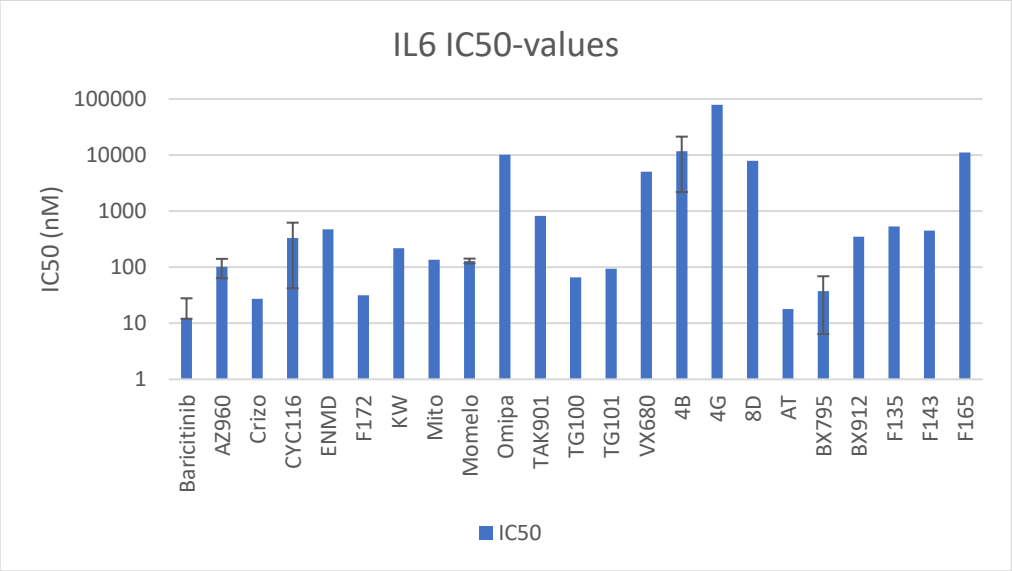

Figure 12. *IC_{50} values (nM) for the slope fit for IL6*

The data in Figure 12 is presented in nM to improve the readability of the table. Some of the IC_{50} values lack standard deviation due to datapoint exclusion described in Section 4.2.4 that were done to improve the reliability of the remaining data and due to the R^2 exclusions. This means that conclusions drawn from the data need to be confirmed with further studies, but the available data can show some general directions. The issues with the data are highlighted by the fact that the two baricitinib-controls used in the two different compound plates the IL6 the experiments were carried out on, show different IC_{50} despite excellent R^2 values. This could however also be due to the fact the cells in the two different plates were from different individuals. All of the plates for all of the individuals had baricitinib as a control, but due to excluded datapoints, it is not possible to confirm if this difference was seen on other replicas.

Although IL6 signals through JAK1/JAK2/TYK2 it has been proposed that JAK1 dominates the pathway (George Abraham et al., 2020). For this reason, IL6 inhibition is regarded in this thesis as inhibition of JAK1. Based on the data presented in Table 16

and in Figure 12, almost all of the inhibitors look promising. Some examples of good sub micromolar inhibitor are Crizo with an IC_{50}-value of 0.027 µM, F172 with an IC_{50}-value of 0.032 µM, TG100 with an IC_{50}-value of 0.066 µM, TG101 with an IC_{50}-value of 0.093 µM and AT with an IC_{50}-value of 0.018 µM.

When the IL6 data is compared with the JAK1 Lance Ultra data, it is shown that from the JAK1 JH2 exclusive binders Omipa shows good IC_{50} value (10 µM) of cytokine signalling inhibition. 4G seems to be a weaker inhibitor with IC_{50} of 78 µM. Both of these were shown to be really poor or no inhibitors on the Lance Ultra data, with IC_{50} values of 120 µM and no inhibition respectively. This means that Omipa and 4G might not inhibit the function of JAK1 through regulation of JH1 by JH2 inhibition. 4G showed inhibition of JAK3 in the Lance Ultra studies, which means that it might not be an exclusive inhibitor of JAK1. Omipa however, showed very little inhibition of JAK3 in the Lance intra studies. It was not possible to determine an IC_{50} value for Picti from the IL6 data due to poor R^2 values. In the Lance Ultra studies it was shown that Picti was a possible activator of JAK1 and this could be one explanation behind the lack of JAK1 inhibition seen in IL6 studies. Due to general issues with the cytokine signalling studies, instead of drawing the conclusion that there is no inhibition for Picti, the studies should be replicated to gain reliable results.

For the compounds that bound to both JAK1 and TYK2 JH2 domain, it was not possible to determine the IC_{50} value for one of them, F7. However, it was possible for Mito, F172 and F165 with the IL6 data, although the R^2 value and standard deviation for Mito was not optimal. The results showed, that both Mito and F165 were able to inhibit the signalling of IL6, even though both of them showed really poor inhibition in JAK1 in the Lance Ultra studies. Mito had an IC_{50} value of 0.14 µM, F172 had an IC_{50}-value of 0.032 µM and F165 had a value of 11 µM in the cytokine studies. All compounds showed inhibition of JAK3 in Lance Ultra studies, so it is possible that these compounds are not as exclusive to JAK1 and TYK2 as could be determined form the binding assays. Nevertheless, the fact that inhibition was seen for three compounds that did not exhibit any JAK1 inhibition in Lance Ultra goes to show that the mechanism of inhibition is not likely JH1 inhibition through JH2 domain with these compounds either.

Compounds screened for JAK3 from set 3, BX795 and BX912 have good IC_{50}-values of 0.038 µM and 0.35 µM respectively in the IL6 data. This means that although the compounds are not exclusive to JAK3, they show potential as inhibitors.

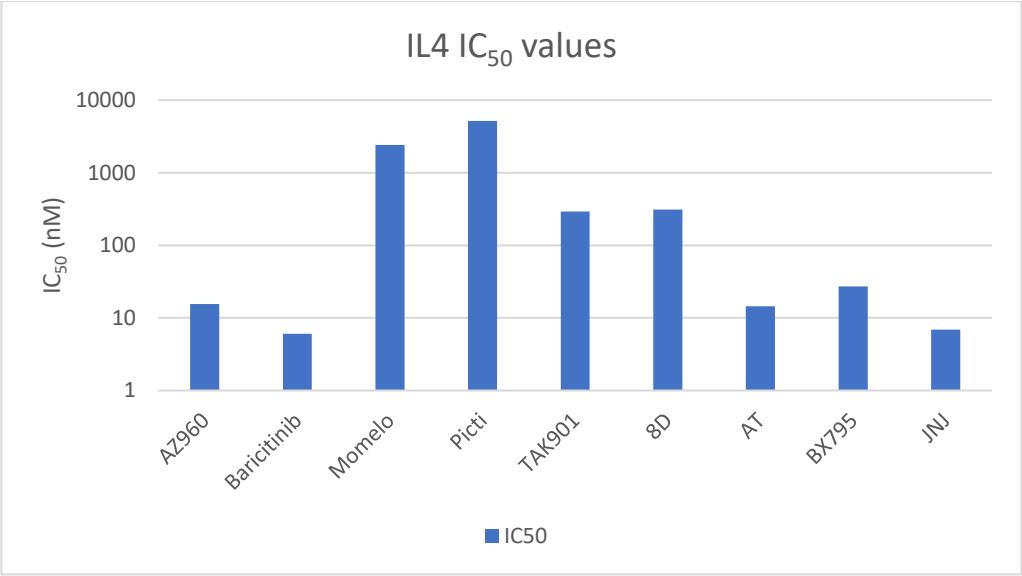

Figure 13. *IC_{50} values (nM) for the slope fit for IL4*

Since IL4 lacked any replicas, it is not possible to calculate standard deviation for the IC_{50} values presented in Figure 13. Furthermore, due to poor R^2 value, it was not possible to establish an IC_{50}-value for the positive control baricitinib, which limits the evaluation of the reliability of the data.

Furthermore, the data can't be directly compared with JAK3 because IL4 signals through both JAK1 and JAK3 (George Abraham et al., 2020). When we compare the cytokine signalling data with both JAK1 and JAK3 Lance Ultra data, it can be seen that there are differences. Picti, which was established as JAK1 JH2 exclusive binder in the binding studies, shows good inhibition of IL4 signalling with an IC_{50} value of 5.2 µM. This is not in line with the Lance Ultra studies, since they showed that Picti didn't inhibit JAK3 and possibly activated JAK1. Since IL4 signals through both JAK1 and JAK3, it is not possible to determine which JAK, is more responsible for the inhibition. Because Picti seemed to possibly activate JAK1 through JH1-2 domains, it is possible that the inhibition is achieved through different mechanisms and the activation is not observed on a cellular level.

BX795 shows good inhibition also in the IL4 data and the levels are similar to IL6 data. Kinase activity studies done previously (not detailed here) show that BX795 inhibits the kinase activity of JAK1, JAK2 and JAK3 equally. Previously done binding JH1/JH2 selectivity studies show that BX795 is selective to JH1 domain for all three JAKs. This means that the similar levels of IL4 and IL6 inhibition are expected and IL4 signalling is also likely inhibited through JAK3 inhibition. This makes BX795 not a selective JAK3 JH2 inhibitor, but it appears to have potential as an inhibitor regardless.

Although an IC_{50}-value could not be established for BX912 in the IL4 studies, previous data shows sub micromolar inhibition of all JAKs in kinase activity assay. Furthermore, JH1/JH2 selectivity studies show that BX912 has nanomolar or sub micromolar binding to all JH1 or JH2 domains with the exception of JAK2 JH2 and JAK3 JH2. No binding was found to JAK2 JH2 and the binding to JAK3 JH2 was micromolar. This could mean that although no IL4 inhibition was seen, this could be only due to unreliable dataset rather than actually non-existing inhibition.

PRT06 was a compound from the set 3 screened for JAK3 JH2. It did not result any IC_{50}-value due to poor R^2 fit. It had been a compound of interest due to binding selectivity to JAK3 JH2 domain. In previous kinase activity studies sub micromolar inhibition was seen for JAK1 and JAK2 as well as micromolar inhibition of JAK3 and TYK2. The lack of inhibition in the cytokine signalling studies could be due to issues with the experiment, so the results should be replicated to gain more reliable data. It is still possible that PRT06 does not inhibit cytokine signalling.

In the IL4 data, it was not possible to determine an IC_{50}-value for Omipa, which showed poor inhibition for both JAK1 and JAK3 in Lance Ultra, but good inhibition with IL6 stimulation. Of the interesting compounds that had good IC_{50}-values in the IL6 studies, the IC_{50} value could not be established also for 4G, Mito and F165 as well as for BX912. This was due to poor R^2 fit with Omipa, Mito, F165 and BX912 and with 4G the large amount of unreliable and thus excluded data points.

6 CONCLUSIONS

In this thesis, the suitability of several potential JAK1/JAK3 inhibitors were assessed. This was done through binding affinity assays and by studying the inhibition of JAK activity and cytokine signalling.

The results showed that Omipa and 4G, which were exclusively binding to JAK1 JH2 with IC_{50}-values of 36 µM and 49.1 µM had showed inhibition of the downstream signalling of IL6 with IC_{50}-values of 10 µM and 78 µM. Picti was missing the IC_{50} value for IL6 inhibition. In the Lance Ultra studies it was shown that Picti possibly activated JAK1, so it is possible that this was the reason behind lack of JAK1 inhibition in the cytokine signalling studies. Picti also showed good inhibition of the downstream signalling of IL4 with an IC_{50}-valu of 5.2 µM, but an IC_{50} value could not be calculated for 4G due to unreliable signal for Omipa due to poor R^2 fit. The cell studies should be replicated to Picti, Omipa and 4G to gain better understanding about their ability to inhibit the signalling of IL6 and IL4 respectively.

Mito and F165, which bind to both JAK1 JH2 and TYK2 JH2, had good inhibition of IL6 signalling, but due to poor R^2 fit the inhibition could not be determined to IL4 signalling. For Mito, the R^2 value and standard deviation were not ideal even for IL6 signalling, so the results should be taken with caution. Since Mito and F165 are not exclusive JAK1 JH2 binders, they do not fulfil the criteria set for potential hits. This might not be an issue, because clinically used inhibitors specific to certain JAKs exhibit some level of inhibition of other JAKs as well. For example, filgotinib, which is a JAK1 specific inhibitor also shows inhibition of JAK2. Furthermore, the inhibition of TYK2 was not assessed in this thesis. This means, that it is still possible that the TYK2 JH2 binding does not translate into TYK2 inhibition.

BX795 and BX912, binders screened for JAK3 JH2 showed inhibition of mainly JAK1 mediated IL6 signalling, so they are not exclusively inhibiting the function of JAK3. Also, based on previous studies BX795 and BX912 do not seem like a selective JAK3 inhibitors. PRT06 from the same compound set had been shown to be an exclusive JAK3 JH2 binder. The cytokine signalling studies did not result in any IC_{50}-values which could indicate that it does not exhibit JAK3 inhibition. These results should be verified through further studies before concluding that PRT06 has no effect on IL6 and IL4 signalling.

Overall, the interesting compounds did not show inhibition in the Lance Ultra studies, but some did in the cytokine signalling inhibition studies. This means that the mechanism of inhibition is likely not inhibition of JH1 activation through JH2, but instead something else like for example prevention of dimerization. This is further supported by the fact that, the only compounds that showed good inhibition in the Lance Ultra studies all had JH1 binding. This is, however, not totally unspringing, because deucravacitinib, a TYK2 JH2 inhibitor in clinical trials is known to be an allosteric inhibitor (H.-O. Kim, 2020) and it seems (although no data has been provided to confirm this) that deucravacitinib achieves this inhibition by stabilizing an autoinhibited conformation of JH2 domain (Glassman et al., 2022). Recent discoveries also suggest that JAK activation by JAK dimerization happens largely through JH2 dimerization (Glassman et al., 2022), which

means that prevention of dimerization is a potential inhibition mechanism for the JH2 exclusive compounds that inhibited cytokine signalling.

All this highlights the importance of studying inhibition of JAK signalling on cellular level. Some form of inhibition assay similar to Lance Ultra might still be needed because it is difficult to establish JAK specificity from cytokine signalling studies due to the dimeric or oligomeric nature of JAKs. By finding out the inhibitory specificity of the compounds, it would be easier to determine which cytokine signalling the compound has the capability to inhibit. This would make it possible to use the compound in targeted manner in disorders where the same cytokines are expressed.

The cytokine signalling experiments used to study cytokine signalling need optimization. The two main issues were unclear barcoding and failure of IL2 stimulation. Since these issues had not been seen in previous experiments done in our lab using fresh PBMC cells, it is possible that the freezing process could be an explaining factor. How this might be is unclear. The freezing process could be optimized by for example using a medium designed to used when freezing cells. For example, CellBanker 2 CPA medium has been shown in a study to be suitable for freezing PBMC cells (Zeng et al., 2020). The barcoding could be optimized by increasing the range of concentrations of dye used and by making sure that the compensations controls are done carefully.

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