Assessment of Tofacitinib and Ruxolitinib and their Anti Inflammatory Effects on Myeloperoxidase

By

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Keywords: Inflammation, Myeloperoxidase, Tofacitinib, Ruxolitinib, and Janus Kinase

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Abstract

Myeloperoxidase (Mpo) is a heme-containing enzyme present in inflammation. Mpo catalyzes the production of hypochlorous acid (HCLO) by reacting with hydrogen peroxide (H$_2$O$_2$) and chloride (Cl$^-$). Mpo together with H$_2$O$_2$ and a halide represents a potent oxidizing system that is involved in a variety of functions that include the killing of bacteria, and the lysis of mammalian cells and inflammatory mediators. To determine this inhibition of mpo we tested inhibitors that we know inhibit. To start, we tested this inhibition by H$_2$O$_2$ dependence. Serial dilutions of H$_2$O$_2$ were made using 30% stock of H$_2$O$_2$ to determine the concentration of H$_2$O$_2$ necessary to produce the greatest fluorescence signal using 530nm excitation and 590 nm emission wavelength using a SpectraMax plate reader. This allowed us to see the greatest fluorescence value for that particular dilution. Fluorogenic peroxidase substrate 10-acetyl-3, 7-dihydrophenozazine (ADHP), MPO, was mixed in sodium acetate buffer at pH 5.6 in the absence and presence of H$_2$O$_2$ to serve as the negative and positive controls, respectively. Three compounds were tested for mpo inhibition, namely benzoic acid hydrazide (bah), tofacitinib, and ruxolitinib. The same conditions were used for each inhibitor. First we tested each inhibitor at a concentration of 10mM. Once we received data we did an one-way ANOVA test. After completing an ANOVA test comparing the positive control to each inhibitor it was determined that both bah and tofacitinib were significantly different but ruxolitinib was not. The concentrations of the inhibitors were 10mM, 5mM, 0.5mM
and 1 mM respectively. The experiment was done again under the same conditions. After completing an ANOVA test and comparing each inhibitor with the positive control again it was determined that all inhibitors were significantly different at 1mM. This information does in fact tell us that all three inhibitors do inhibit mpo. After testing these inhibitors at different concentrations we determined the solubility of them is a factor, potentially a limiting factor in the inhibition of mpo. Further tests will be done to see what effect does the solubility have on mpo inhibition, if there is an effect.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2-ABAH</td>
<td>2- amino benzoic acid hydrazide</td>
</tr>
<tr>
<td>ADHP</td>
<td>10-acetyl-3, 7-dihydroxyphenoxazine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>one-way analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAH</td>
<td>benzoic acid hydrazide</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>chloride ion</td>
</tr>
<tr>
<td>CIA</td>
<td>collagen induced arthritis</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>DMARD</td>
<td>disease modifying anti rheumatic drug</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EPO</td>
<td>eosinophil peroxidase</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FDA</td>
<td>food and drug administration</td>
</tr>
<tr>
<td>HOCl</td>
<td>hypochlorous acid</td>
</tr>
<tr>
<td>H(_2)O(_2)</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>IL</td>
<td>interleukins</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>LPO</td>
<td>lactoperoxidase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>MQ</td>
<td>milli q water</td>
</tr>
<tr>
<td>MTX</td>
<td>methotrexate</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cells</td>
</tr>
<tr>
<td>NETs</td>
<td>neutrophil extracellular traps</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH oxidases</td>
</tr>
<tr>
<td>O₂</td>
<td>superoxide anion</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear neutrophils</td>
</tr>
<tr>
<td>ROS/RNS</td>
<td>reactive oxygen and nitrogen species</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>TPO</td>
<td>thyroid peroxidase</td>
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Chapter 1 Literature Review

1.1 Introduction

Myeloperoxidase (Mpo) is a heme-containing enzyme in inflammation. It is a major enzyme in innate immunity and defense against pathogens. The expression of mpo expression is limited to myeloid cells, and its synthesis in neutrophils starts at the promyelocyte stage and terminates at the beginning of the myelocyte stage [1]. Mpo is also present in monocytes but at a lesser extent. Mpo is abundant, accounting for 5% of dry weight in neutrophils and present in a lesser extent in other myeloid cells like monocytes and macrophages [1]. Neutrophils are implicated in the tissue damage that occurs in a variety of disease states including rheumatoid arthritis (RA). During phagocytosis polymorphonuclear leukocytes (PMNs) undergo a series of respiratory bursts and electron transfers which gives way to oxygen being reduced to the superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$). Mpo catalyzes the production of hypochlorous acid (HOCl) by utilizing chloride (Cl$^-$) and H$_2$O$_2$, predominately generated by NADPH oxidase [13]. HOCl is a very powerful neutrophil oxidant that is cytotoxic. Although H$_2$O$_2$ alone is microbicidal, its bactericidal activity is constituted by mpo. Mpo together with H$_2$O$_2$ and a halide represents a potent oxidizing system that is involved in a variety of functions that include the killing of bacteria, and the lysis of mammalian cells and inflammatory mediators. Mpo has a mass of 140–155 kDa. Its biosynthesis is a complex
process including proteolytic events, heme and glycan additions, and a final dimerization step [1,2]. Nascent mpo, called preproMPO, undergoes a first proteolytic event and N-glycan addition to make apoproproMPO in the endoplasmic reticulum (ER). This forms proMPO, which leaves the ER and travels to the Golgi apparatus and granules where mpo undergoes several new proteolytic events.

In the azurophilic granules, mpo is kept in an inactive state as long as the neutrophil is not activated and hydrogen peroxide (H₂O₂) is absent [3]. Activation of neutrophils leads to the release of the contents of the azurophilic granules (including mpo) into phagosomes and to the assembly of the NADPH oxidase enzyme complex (NOX2) that produces superoxide radicals (O₂) after phagocytosis. This radical is highly reactive and unstable and is converted rapidly into H₂O₂ spontaneously or by the action of superoxide dismutase H₂O₂, which has a lower oxidation potential, can reach the ingested pathogen and contributes to its destruction by oxidizing vital molecules [5]. The reactivity of H₂O₂ alone does not produce optimal antimicrobial efficacy. Mpo can also use other (pseudo-) halide anions including Bromide (Br⁻) Iodine (I⁻) and thiocyanate (SCN⁻) to give the corresponding hypo- (pseudo-) halogenous oxidants [4]. The first reaction of mpo is its oxidation by H₂O₂ to give Compound I. In the halogenation cycle, mpo is then reduced back to its native form in a two-electron reaction. The latter enables the generation of hypo- (pseudo-) halogenous acid. Although Cl⁻ has the lowest reactivity to mpo among (pseudo-) halide anions [6], it is considered to be the major physiological substrate of mpo due to its high in vivo concentration [7-10]. HOCl is a strong oxidant, and it is
thought to be more efficient than H$_2$O$_2$ in killing pathogens [11]. Due to its powerful oxidation products, mpo would be required to give the neutrophil optimum antimicrobial activity. Although neutrophils retain normal phagocytosis activity when mpo is inhibited or deficient, they cannot kill all types of ingested pathogens [12]. Despite its key role in host defense, mpo has also been involved in pathologic states. Indeed, during chronic inflammation or acute oxidative stress, mpo is released into the extracellular space where oxidants can be produced and host tissues damaged [4].

1.2 Importance of Myeloperoxidase

The significance of mpo is evident from studies on people with total or incomplete mpo deficiency [3]. The MPO/HOCl system plays an important role in intracellular microbial killing by neutrophils [12]. However, mpo can also be released extracellularly after the activation of leukocytes, which can cause tissue damage along with the formation of reactive intermediates. Because of this, mpo has been implicated in the progression of a number of disease states including atherosclerosis, cardiovascular disease, kidney disease, cystic fibrosis and multiple sclerosis [12]. This provides evidence that would implicate mpo as an important therapeutic target in the treatment of inflammatory conditions. A role of mpo as a local mediator of tissue damage has also been demonstrated in models of cardiovascular, renal and lung diseases [12]. Evidence has also been given that shows mpo in the pathogenesis of RA as a local mediator of joint damage particularly in RA. Mpo is released by activated neutrophils in RA synovial fluid, where enhanced levels of enzymatically active mpo correlate with the presence of
HOCl- modified proteins [12]. Increased concentrations of active mpo were also found in the inflamed joints of mice in previous studies that had RA.

1.3 Neutrophils and their relation to MPO

The PMNs, or neutrophils have for a long time been considered as the primary soldiers of the innate immune system. As the body main infantry, they are the most abundant fraction of white blood cells in mammals [15]. Neutrophils are polymorphonuclear granulocytes that carry storage granules filled with proteins and enzymes. Neutrophils account for 50-70% of leukocytes in humans. Neutrophils are the first leukocytes to respond under acute inflammation migrating to sites of damage to sanitize the area by killing microorganisms through phagocytosis. These cells have long been thought of as short-lived cells of the innate immune response [14]. However, recent research evidence has demonstrated that neutrophils persist beyond acute inflammation to initiate and perpetuate chronic inflammation. The onset of inflammation increases the lifespan of neutrophils in circulation, anywhere from 12 h to several days [16]. Neutrophils bring together both the innate and adaptive immune response in autoimmune disease.

Neutrophils are terminally differentiated cells with a short lifespan in circulation. As a first line of defense against invading microorganisms, neutrophils are characterized by their ability to act as phagocytic cells, release lytic enzymes from their granules and produce reactive oxygen species. The neutrophil-mediated inflammatory response is a multistep process, initially characterized by adhesion of granulocytes to the activated
vasculature. Neutrophils engage in complex bidirectional interactions with macrophages, dendritic cells, natural killer cells, lymphocytes and mesenchymal stem cells. Several innate and adaptive immune cells can modulate neutrophil function. Neutrophils are characterized by two morphological characteristics: the shape of their nucleus and their granules, which provide sequential release of bactericidal proteins into the extracellular space [17]. Granules are classified into four groups: primary or azurophilic, secondary or specific, tertiary and secretory vesicles.

Neutrophils can also immobilize pathogens extracellularly by releasing neutrophil extracellular traps (NETs) [18]. During NET formation, neutrophils may die through a distinct cell death program termed NETosis [21]. To limit potentially excessive inflammatory responses, neutrophils are characteristically short-lived and die in circulation within 4 to 10 hours. However, the neutrophil lifespan can increase in response to cytokines or other proinflammatory agents [19]. Furthermore, recent data from in vivo labeling of human neutrophils with deuterium suggests a considerably longer half-life, averaging around 5 days in circulation, while murine neutrophils have a significantly shorter half-life [20]. Over the last several years, a renewed interest in the role that neutrophils play in various systemic autoimmune diseases has emerged.

The identification of increased neutrophils in RA synovial fluid in early disease stages supports a role for these cells in the pathogenesis of joint destruction. Activated neutrophils have been found in RA synovial fluid, synovial tissue and RA-associated skin disease [21-24]. Remission of RA has been linked with changes in neutrophil adhesion.
Circulating and synovial fluid RA neutrophils are more prone to form NETs when compared with neutrophils from healthy controls and from patients with osteoarthritis [18].

1.4 Rheumatoid Arthritis and Inflammation pertaining to MPO

Rheumatoid arthritis (RA) is a symmetric polyarticular arthritis that primarily affects the small diarthrodial joints of the hands and feet. It is an autoimmune disease, in which the body generates antibodies against its own tissues [14]. It is a disease that damages the joints of the body [32]. Adaptive and innate immunity contribute to the development of RA. CD4+ T cells, B cells, macrophages and neutrophils are some of the cells that accumulate in the joints. This gives way to the development of the disease.

Many variables are recorded to monitor the disease progression. It is characterized by tissue-specific autoimmune-mediated chronic inflammation that affects multiple joints and results in destruction of cartilage and bone loss. Risk factors of RA are multi-dimensional and include genetic defects, infections, and environmental influences[25]. About 1% of the general population has RA, and many patients develop long-term joint damage, severe illness, and disability [43]. The mechanisms underlying RA are complex, including genetic and environmental factors, inflammatory cytokines, and abnormalities of both innate immunity and adaptive immunity [44,45].

Immune and inflammatory responses are the driving forces in RA and transform the synovial membrane into an inflammatory tissue capable of invading and destroying
adjacent cartilage and bone [33, 34]. Cytokines, regulators of immune and inflammatory responses, are involved in both innate and adaptive immunity, and also implicated in the pathogenesis of RA. Neutrophils account for the majority of inflammatory cells in the synovia of human RA patients, and the joints of collagen-induced arthritis (CIA) mice, a murine model of RA [26,27]. In both humans and mice, neutrophils accumulate at the pannus-cartilage interface, where much the destruction to both bone and cartilage takes places [28–31].

The inflamed synovium is infiltrated by neutrophils, macrophages, T cells and B cells, which release a variety of pro-inflammatory mediators [35]. Persistent inflammation results in destruction of cartilage and bone. This occurs through a number of mechanisms, including oxidative and proteolytic breakdown of collagen and proteoglycans [36]. Once sequestered within the joint space, neutrophils degranulate and release a variety of potentially harmful enzymes and peptides [34]. HOCl is the major strong oxidant generated by neutrophils when they kill bacteria [38] and it is also produced at sites of inflammation. It reacts predominantly with methionine and cysteine residues in proteins to disrupt their tertiary structure as well promote intra- and inter-molecular cross-links and inactivate enzymes [39]. RA is a heterogeneous disease, in which mpo may play a role in the pathogenesis, severity and/or outcomes [37]. Mpo is present at high concentrations in SF of patients with RA. In apparently healthy individuals, plasma mpo concentrations predict the presence of coronary artery disease as well as future risk of coronary artery disease [37] Cardiovascular disease (CVD) is
recognized as an important cause of death in patients with RA [40]. The link between inflammation and cardiovascular disease is further supported by evidence that diseases modifying anti-rheumatic agents such as methotrexate (MTX), which suppress the inflammatory process in RA, are associated with lower cardiovascular mortality rates in patients with RA [41, 42].

1.5 JAK/STAT Pathway and how it works with inflammation

Cytokines are soluble factors with critical functions in the immune system. In particular they serve as an intracellular communication tool of immune system, and their release and actions help shape the immune response [45]. As a result, when these molecules are produced in abnormal amounts, the homeostasis of the immune system is altered and several pathologies ensue. Autoimmune disorders are a classical example of such pathologies as several pro-inflammatory cytokines have been demonstrated to drive such diseases [46]. It came as no surprise that targeting cytokines and their receptors resulted in the development of several drugs currently utilized to treat autoimmune diseases. The class of drugs known as biologics which includes monoclonal antibodies, recombinant soluble receptors and fusion proteins of receptor moieties with antibodies constant fragments have, in the past 15 years, completely revolutionized the clinical approach to the treatment of immune disorders [47].

The activation of the tyrosine kinase of the Janus family, better known as JAKs, was shown to be a critical step. This family comprises four molecules namely JAK1, JAK2,
JAK3 and TYK2 [45]. JAK pathways are normally involved in growth, survival, development and differentiation of a variety of cells, but are crucially important for immune and hematopoietic cells [51]. Each JAK protein has specificity for a different set of cytokine receptors; the function of the JAK protein is thereby linked to the function of the cytokines that bind the receptors [8, 11]. Each cytokine receptor requires at least two associated JAKs in order to signal[51]. JAKs may work in pairs of identical JAKs (e.g. JAK2/JAK2) or of different JAKs (e.g. JAK1/JAK3)[51]. JAK3 is the most specific, associating with only the common γ-chain (γc) receptor subunit and JAK1. JAK1 associates with the receptors for IFNs and IL-10-related cytokines, γc cytokines, IL-6, as well as other cytokine receptors containing the gp130 subunit [52, 53]. It forms pairs with any of the three other JAKs. Finally, TYK2 transmits signaling by type I IFNs (IFNα, IFNβ), IL-12 and IL-23, amongst others [8, 11]. Binding of a cytokine to its receptor activates the receptor-associated JAKs [54]. The activated JAKs phosphorylate specific tyrosine residues in the cytoplasmic domains of the cytokine receptor subunits, which then act as docking sites for Signal Transducer and Activator of Transcription (STAT) proteins [12, 13]. The STAT family of transcription factors consists of seven proteins: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 [12]. After docking to tyrosine-phosphorylated cytokine receptor subunits, the STATs themselves are, in turn, tyrosine-phosphorylated by the receptor-associated JAKs [12]. Phosphorylated STATs then dissociate from the receptor subunits, combine with each other, and translocate to the cell nucleus. In RA, B cells, T cells, macrophages and other leukocytes infiltrate the
synovium in response to pro-inflammatory cytokines and chemokine’s, leading to inflammation and tissue destruction [51].

Upon binding of cytokines to their cognate receptors, JAKs, which work in pairs, become enzymatically active and phosphorylate themselves, the receptor chains, and several other substrates including the Signal Transducers and Activation of Transcription (STATs) family of latent transcription factors [50]

JAK inhibitors can be used in both immunosuppression and to suppress hematopoiesis. Shown here are three cytokines and their cognate receptor complexes with the associated JAKs. IL-2 and EPO receptor activation predominantly recruits STAT5, whereas IFNγ receptor activation mainly results in STAT1 recruitment. The recruited STATs are activated via phosphorylation by the associated JAKs, after which they dimerize and translocate to the nucleus where they regulate transcription.

1.6 Mechanism of Janus kinase inhibitor Tofacitinib

Tofacitinib (CP690, 550, Xeljanz; Pfizer) was initially designed to be a specific inhibitor of JAK3 kinase and therefore intended primarily to be used as an immunosuppressant in transplantations and for the treatment of autoimmune diseases [56]. Tofacitinib is an oral JAK inhibitor for the treatment of RA. It is a targeted synthetic small molecule (molecular weight 312.4 Da; 504.5 for the citrate salt), not a biologic [51]. Unlike targeted biologic therapies, tofacitinib works at the cellular level blocking JAK3. More recently, it was found that tofacitinib also inhibits the kinase activity of the
JAK1 enzyme but has little effect on JAK2 or TYK2 function [57,58]. However, inhibiting JAK1 is beneficial in immunosuppression [56] Similar to ruxolitinib, tofacitinib can also be administered orally. However it received approval from the Food and Drug Administration (FDA) for patients who have moderate to severe RA for which MTX did not work. Tofacitinib can be used as a monotherapy or in combination with MTX and/or other non-biological disease modifying antirheumatic drugs (DMARDs). Tofacitinib possesses high in vitro passive permeability properties consistent with intracellular entry by transcellular diffusion. Tofacitinib is a reversible, competitive inhibitor that binds to the adenosine triphosphate (ATP) binding site in the catalytic cleft of the kinase domain of JAK (51). The structure of tofacitinib mimics that of ATP without the triphosphate group. As a result of binding to the ATP site, tofacitinib inhibits the phosphorylation and activation of JAK, thereby preventing the phosphorylation and activation of STATs, and thus the activation of gene transcription. This leads to decreased cytokine production and modulation of the immune response. Tofacitinib is a potent inhibitor of the JAK family of kinases with a high degree of selectivity against other kinases in the human genome. With in vitro kinase assays, tofacitinib inhibits JAK1, JAK2, JAK3 and, to a lesser extent, TYK2. In cellular settings, where JAKs signal in pairs, Tofacitinib preferentially inhibits signaling by cytokine receptor associated with JAK3 and/or JAK1 with functional selectivity over receptors that signal via pairs of JAK2 [52]. By inhibiting JAKs, tofacitinib may alter leukocyte recruitment, activation, and effector cell function at sites of inflammation. In fact, in the case of tofacitinib, the
capacity to inhibit the actions of several pro-inflammatory cytokines and to act on different immune cells is possibly the reason why this drug has been so efficacious in RA, a disease which pathophysiology involves the action of several cytokines (45).

In the case of tofacitinib, the total number of circulating T cells is not impaired but differentiation of T helper (Th) cells such as Th1, Th2 and Th17 is impaired [48]. Animal studies have also shown a sharp decline in numbers of NK cells. Patients treated with tofacitinib tended to be more prone to infections, which included opportunistic pathogens and herpes zoster. The above-mentioned effect on NK cells does not appear to correlate with increase incidence of tumors but long terms effects have not yet been evaluated.

Under normal circumstances, binding of a cytokine to its specific cell-surface receptor causes the receptor chains to polymerize and activate the associated JAKs. Activated JAKs phosphorylate specific residues in the cytoplasmic domains of the cytokine receptor chains, which then act as docking sites for STAT proteins. Once they have docked, STATs are phosphorylated by the activated receptor-associated JAKs. Phosphorylated STATs then dissociate from the receptor chains, dimerize with each other, and translocate to the cell nucleus where they activate gene transcription. Tofacitinib binds in the catalytic cleft in the kinase domain of JAK. This prevents activation of JAK and STAT phosphorylation and translocation to the nucleus to activate gene transcription.
1.7 Myelofibrosis and Ruxolitinib

Myelofibrosis is a disease characterized by marrow fibrosis, extra medullary hematopoiesis, splenomegaly, leukoerythroblastic blood picture, elevated levels of peripheral blood CD34 cells, and myelofibrosis-related symptoms, such as abdominal discomfort, pain under the left ribs, night sweats, pruritus, bone or muscle pain, and early satiety [1]. Myelofibrosis, including primary myelofibrosis, post polycythemia Vera myelofibrosis, and post essential thrombocytemia myelofibrosis, is a chronic disease affecting primarily older patients [2, 3]. The median overall survival is 11.3 years for low-risk, 7.9 years for intermediate-1 risk, 4.0 years for intermediate-2 risk, and 2.3 years for high-risk myelofibrosis [4].

Ruxolitinib (trade name Jakafi) is a JAK2/JAK1 inhibitor (with some activity on JAK3 and TYK2) currently prescribed for the treatment of intermediate or high-risk myeloproliferative disorders including primary myelofibrosis post-polycythemia vera myelofibrosis and post-essential thrombocytemia myelofibrosis. Ruxolitinib is also a small molecule inhibitor of JAK1 and JAK2 and it inhibits the binding of ATP to JAKs. Due to the central role of JAK1 and JAK2 in the regulation of immune responses, ruxolitinib has also been studied in the treatment for autoimmune diseases [42]. Administration of a JAK2 inhibitor like ruxolitinib results in anemia and thrombocytopenia as expected by the well-known role of JAK2 in erythropoietin and thrombopoietin signal transduction.
1.8 Concluding Remarks

Studying these inhibitors is not a new topic. The literature has shown that there have been studies on Janus kinase inhibitors. However the safety and efficacy of these two inhibitors, tofacitinib and ruxolitinib do deserve some thought. Other inhibitors of mpo and Janus Kinase have been identified but what’s so fascinating about tofacitinib and ruxolitinib is that they both received FDA approval for two different disease states but their structures are very similar. So this did deserve a second look. Janus Kinase and JAK/STAT pathways do play a role in mpo inhibition. We decided to test these inhibitors to see if they do inhibit mpo and if so to what extent. We want to look at what concentration(s) these Janus Kinase inhibitors have an effect on mpo. Some concentrations will have a greater effect than others. Other psychological properties may play a role in the concentration like solubility, pka, etc. Once we determine what concentration is the inhibition the greatest we can conduct studies using murine models and the zebra fish model. Many studies have been done using the zebra fish model especially in the developmental phase. These studies will provide insight on the results we could potentially receive in human studies.
Chapter 2: Evaluating Janus Kinase Inhibitors and their effect on MPO Inhibition

2.1 Introduction

Myeloperoxidase (Mpo) has a prominent role in chronic inflammatory diseases particularly RA. Mpo is a heme protein derived from neutrophils, monocytes, and macrophages. Neutrophils are present at large amount while monocytes and macrophages are present in a lesser extent. Mpo is a member of the superfamily of mammalian hemeperoxidase enzymes, which also includes eosinophil peroxidase (EPO) and lactoperoxidase (LPO) [58]. Generally, all peroxidases have an active-site heme group that contains a central iron atom that maintains its oxidation state (i.e., reactivity) through coordination with a distal His residue [67]. In its resting state, mpo contains ferric heme (MPO-Fe (III)). Mpo has a ferric heme, MPO-Fe (III), which is oxidized to a short-lived intermediate, termed Compound I by reacting with a 100–10,000-fold lower relative concentration of H$_2$O$_2$, which contains a ferryl porphyrin π cation radical (Eq. 1) [68, 69]. In the absence of Cl$^-$ and in the presence of classical peroxide electron donor (AH$_2$), mpo follows a typical peroxidase catalytic cycle where Compound I is reduced back to the ferric state in two sequential one-electron steps (Eq. 2-3). [67] The porphyrin radical is reduced to a ferryl heme, known as Compound II, in the first step (Equation 2)[67]. Compound II then is reduced back to ferric enzyme A by AH$_2$ in step (Equation 3). At the same time, AH$_2$ is oxidized to the free radical (AHd) [70, 71]
MPO-Fe (III) + H₂O₂ → MPO-Fe (IV) = O⁻ + π + H₂O (Eq. 1)

“Compound I”

Compound I + AH₂ → MPO-Fe (IV) = O + AH (Eq. 2)

“Compound II”

Compound II + AH₂ → MPO-Fe (III) + AH + H₂O (Eq. 3)

Compound I + Cl⁻ → MPO-Fe (III) + HOCl (Eq. 4)

MPO-Fe (III) + O₂
− → MPO-Fe (III) O₂
− (Eq. 5)

“Compound III”

In the presence of Cl⁻, MPO Compound I is able to oxidize Cl⁻ to HOCl, and in the process Compound I is reduced directly to the ferric state (Eq. 4). Neither Compound II (Eq. 3) nor superoxide-inactivated Compound III (Eq. 5) participates in Cl⁻ oxidation. These reactions (Equations 1-5) occur through octahedral coordination of the active site Fe by the protoporphyrin IX heme and the proximal histidine 336 on the mpo heavy chain (HCHis336). With mpo having such an important role in the inflammatory responses in chronic inflammatory disease it is worth noting that mpo is present at a high concentration at the site of inflammation. In humans, mpo has become a biomarker for heightened inflammation because of its role in the production of oxidized DNA and protein adducts [59]. For inhibitory purposes mpo follows either a one step or two step mechanism.
E + S $\leftrightarrow$ ES : $k_{+1}$ $k_{-1}$
ES $\rightarrow$ E + P : $k_2$
E + I $\leftrightarrow$ EI : $k_{+3}$ $k_{-3}$

Scheme I. The one-step model tested.

E + S $\leftrightarrow$ ES: $k_{+1}$ $k_{-1}$
ES $\rightarrow$ E + P : $k_2$
E + I $\leftrightarrow$ EI : $k_{+3}$ $k_{-3}$
EI $\leftrightarrow$ EI* : $k_{+4}$ $k_{-4}$

Scheme II. The two-step model tested.

Where E is the enzyme, S is the substrate, and I is the inhibitor. $k_{+1}$, $k_{-1}$, $k_2$, $k_{+3}$,$k_{-3}$, $k_{+4}$, and $k_{-4}$ are the rate constants. For example, ROS and RNS production during oxidative stress has been linked to heightened Mpo levels in chronic obstructive pulmonary disease [60], and RA [61]. Mpo is a critical mediator in joint inflammation and tissue damage in chronic diseases.

Rheumatoid Arthritis is an autoimmune disease in which the body’s immune system attacks the joints. It is characterized by synovial and systemic inflammation. The inflamed synovium is infiltrated by neutrophils, macrophages, T cells and B cells, which release a variety of pro-inflammatory mediators [63]. RA is an inflammatory rheumatic disease resulting in severe pain, disability and mortality. Doctors have been studying the cause of RA but they don’t fully understand it. While its not fully understood one thing is for certain it does cause an abnormal response in the immune system, which leads to joint inflammation and variety of other symptoms. The onset of the disease is not similar in all patients. The course of disease can also vary from patient to patient. Early detection can potentially dampen the symptoms associated with this disease. Persistent
inflammation results in destruction of cartilage and bone. This occurs through a number of mechanisms, including oxidative and proteolytic breakdown of collagen and proteoglycans [62-65]. In rheumatoid arthritis, the synovial membrane becomes infiltrated with various inflammatory cell types, which synergize to cause joint destruction. It is clear that T cells are important, as evidenced by the genes associated with rheumatoid arthritis [62]. Macrophages are also vital and their presence correlates with symptoms, perhaps caused by secretion of crucial pro inflammatory mediators.

Once sequestered within the joint space, neutrophils degranulate and release a variety of potentially harmful enzymes and peptides [63]. They may also undergo a respiratory burst and generate several reactive oxygen species, including superoxide, hydrogen peroxide, hypohalous acids, and possibly hydroxyl radical [62, 66]. Although these destructive oxidants have often been held partly responsible for joint destruction, compelling evidence that they are in fact produced within the synovium is lacking.

The transmission of signals from extracellular stimuli across the plasma membrane via the cytoplasm to the nucleus in eukaryotes principally relies on the post-translational protein modification, phosphorylation [72]. Due to their roles in extracellular signaling, protein kinases are subjected to many levels of positive and negative regulation to ensure fidelity of signals and restrict signal longevity to guard against aberrant signal activation. Proliferative disease like cancer are due to defective kinase activity, which gives notice to these kinases at therapeutic targets. Due to their essential roles as signal transducers downstream of cytokine receptor
activation, the Janus Kinase (JAK) family of tyrosine kinases have garnered much attention since their discovery more than 20 years ago [73–78]. This family comprises four members: JAK1, JAK2, JAK3 and TYK2. In contrast to receptor tyrosine kinases, such as the c-Kit and Insulin receptors, cytokine receptors lack intrinsic protein kinase domains and consequently rely on the catalytic activities of constitutively associated Janus kinase (JAK) family of tyrosine kinases to convey signals [72].

Cytokine receptors, each associated with a JAK monomer, comprise two or more receptor subunits, activated JAKs phosphorylate tyrosines within the cytoplasmic regions of the receptor with which they are associated, generating docking sites for downstream adaptor and effector (“reader”) proteins that contain phosphotyrosine recognition domains, typified by its SH2 domain, including the signal transducers and activators of transcription (STAT) proteins [72]. Depending on the receptor, and the docking sites generated by tyrosine phosphorylation within the cytoplasmic region, any one or more of six STAT family members (STAT1, STAT2, STAT3, STAT4, STAT5 or STAT6) may be recruited via their SH2 domains. STATs exist as preformed dimers [79–81], and by being brought into proximity of the receptor-associated JAK can then be phosphorylated by JAK, leading to a reorientation of subunits within the STAT dimer and translocation into the nucleus where it functions as a transcription factor [82–84]. The resulting transcriptional program dictates whether the cell undergoes proliferation, differentiation, survival or death [72].
Tofacitinib citrate (Xeljanz) is an oral, small molecule drug used to treat adults with moderate-to-severe, active rheumatoid arthritis for which methotrexate did not work. Methotrexate is a disease-modifying antirheumatic drug (DMARD) used to treat rheumatoid arthritis (RA). However most physicians aren’t taking the DMARD approach anymore. Tofacitinib acts to block the body’s production of enzymes called Janus kinases (JAKs). JAKs play a role in joint inflammation in RA. By inhibiting JAKs, tofacitinib may modulate leukocyte recruitment, activation, and effector cell function at sites of inflammation. Tofacitinib is currently being studied for use in treating other autoimmune diseases, including psoriasis, psoriatic arthritis, ulcerative colitis, Crohn’s disease and ankylosing spondylitis. However our main concern is its role in the inhibition of mpo and RA.

Ruxolitinib is used to treat certain bone marrow disorders Myelofibrosis. Myelofibrosis is a disease characterized by marrow fibrosis, extra medullary hematopoiesis, splenomegaly, and leukoerythroblastic blood picture, elevated levels of peripheral blood CD34 cells. The pathogenesis of myelofibrosis is not well understood, but appears to involve the activation of the Janus-activated kinases (JAK)/STAT pathway. Ruxolitinib is an inhibitor of JAK1 and JAK 2, inhibits the binding of ATP to JAKs. Though there is not a cure for these disorders, ruxolitinib may help with some of the symptoms, including abdominal discomfort, pain under left ribs, early feelings of fullness from meals, night sweats, itching, and bone/muscle pain.
The goal of this study was to determine (1) if tofacitinib and ruxolitinib inhibit mpo (2) if they do inhibit mpo by what mechanism. We know there is a relationship between mpo and inflammation but what about Jak Kinase? Jak Kinase does play a role in inflammation as well which is why we took notice in these Jak inhibitors. Could Jak inhibitors be MPO inhibitors as well? Another compelling thing was that the Jak inhibitors that we are studying, tofacitinib and ruxolitinib are very similar in structure. Could their mechanism pertaining to the inhibition of mpo be similar as well? We decided to take an in-depth look at this in mpo by doing a fluorescence assay and light scatter experiments.
Figure 2.1 Identification of Myeloperoxidase inhibitors. Tofacitinib and ruxolitinib have been assessed for the ability to inhibit mpo. The purine heterocyclic aromatic ring is one of the noticeable similarities and one of our reasons to study these two inhibitors and their inhibition of mpo. The basic pkas are 7.13 and 5.51 respectively. There is a reported solubility difference when the freebase or salt versions of each compound is used at pH 5.6 in the peroxidase assays.
Figure 2.2 Biology of PMNs in response to injury and inflammation.

Localized overexpression of E-selectin and P-selectin on activated endothelial cells slow the PMN roll upon the endothelium via leukocyte-derived L-selectin. Responding PMNs transmigrate through the endothelial cells after LFA-1 hooks intercellular adhesion molecule 1 (ICAM-1) and arrive at the site of damage just before diapedesis caused by the increased vascular permeability from histamine released from the mast cells. PMNs undergo phagocytosis of the invasive microbes once they arrive at the infection site. In addition, mpo is secreted from the patrolled PMNs to produce the potent antimicrobial reagent HOCl in response to infection.[67] This figure was taken from Huang et al 2016.
2.2 Materials and Methods

2.2.1 Materials

Myeloperoxidase (Mpo) was purchased from Lee Biosolutions Inc. (St. Louis, MO). 2 amino benzoic acid hydrazide (2-ABAH) and sodium acetate were purchased from Sigma Aldrich (St. Louis, MO). 10-acetyl-3, 7-dihydroxyphenoxazine (ADHP) was purchased from ABD Bioquest Inc. Sodium Azide(NaN₃), dimethyl sulfoxide (DMSO) were obtained from Alfa Aesar (Ward Hill, MA). Ruxolitinib freebase, ruxolitinib phosphate, tofacitinib citrate, and tofacitinib freebase were purchased from LC Laboratories(Woburn, MA). Hydrogen peroxide (H₂O₂) was purchased from BDH Chemicals, (London, UK). Cuvettes were purchased from VWR (Secaucus, NY). Three hundred eighty four well plates were purchased from Perkin Elmer(Waltham, Ma). DPBS (Dulbecco’s Phosphate Buffered Saline) was purchased from Thermo Fisher(Grand Island, NY).

MEBSS buffer (144 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl₂, 800 µM MgSO₄, 800µM NaH₂PO₄, 4 mM Hpes, 5.6 mM glucose, pH 7.4 with 1% fetal bovine serum) was used in the luminescence assay. The assay buffer (sodium acetate buffer) was prepared by adjusting the pH of sodium acetate buffer to 5.6 with acetic acid. Working solutions of H₂O₂ were made fresh daily by diluting 30% H₂O₂ according to the extinction coefficient for H₂O₂ at 240 nm, 39.4 M⁻¹cm⁻¹.Tofacitinib citrate, tofacitinib free base, ruxolitinib phosphate, ruxolitinib free base, and bah were dissolved in DMSO and diluted into assay buffer.
2.3 Methods

2.3.1 Mpo Fluorescence Assay

Mpo activity was measured using a number of parameters. This assay was used to study the inhibitory effect of tofacitinib, ruxolitinib and bah on mpo activity. Based off previous studies we know that bah does inhibit mpo so we wanted to see if the mechanism of tofacitinib and ruxolitinib was similar to that of bah which is why we included it in the assay. Spectramax GeminiXPS plate reader (Molecular Devices, CA, USA) with excitation of λ530nm and emission of λ590nm was used in this assay. Reactions of adhp (27 µM) were incubated with mpo (12.4 nM) and titrated tofacitinib in assay buffer (100mM) along with 30% H2O2. Each compound was incubated for 20 minutes at 25°C prior to use. Ten serial dilutions were done using 200µl of 30% H2O2 and 800µl of MQ water. Each dilution was vortexed after and tips were not changed in between dilutions. Three replicates were used for the control (assay buffer, adhp, and mpo) and the standard (assay buffer, adhp, H2O2, and mpo. The control included 28µl of assay buffer, 8µl of adhp and 4µl of mpo for a total volume of 40µl. The standard included 8µl of assay buffer, 8µl of adhp, 16µl of H2O2 and 8µl of mpo. The standard also had a total volume of 40µl. The 384 well plate was centrifuged at 25°C for 5 minutes with a relative centrifugal force (RCF) of 1008. Once we determined the greatest intensity value based off of the H2O2 dilution we used that particular dilution in the next step. The fluorescence intensity endpoint reading was performed after incubation for 5
min. SoftMax Pro software 4.7 parameters included auto-mix time 5 s and auto-calibrate on. For this next step the parameters did change. We now have two controls (negative and positive) and three standards (bah, tof, rox). Its worth noting that the standards did change depending on what inhibitor we were testing (rox free base, rox phosphate, NaN₃, tof citrate, bah). The negative control included 28µl assay buffer, 8µl of adhp, and 4µl mpo. The positive control included 8µl assay buffer, 8µl adhp, 16µl H₂O₂ from the highest dilution in the previous step and 4µl mpo. The parameters for the inhibitors included 8µl of the inhibitor, 8µl of adhp, 20µl of H₂O₂, and 4µl of mpo. All samples had a total volume of 40µl. Graphpad software (version seven) (La Jolla, CA, USA) was used to make the plots.

2.3.2 Luminescence Assay

A ninety-six well plate was used with four inhibitors (tof citrate, tof free base, rox phosphate and rox free base) and three buffers (MEBSS buffer, DPBS, and Assay buffer). Column one contained 300µl of the inhibitor (50mM) and a 1:10 dilutions were performed for columns 2-8. The plate was then incubated for 24 hours at 25°C. SpectraMax Gemini 340PC was to obtain the absorbance of each inhibitor and read at a wavelength of 600 nm. The parameters included auto mix 5s and auto calibrate on. Graphpad software was used to make the plots used.
2.4 Results

2.4.1 H$_2$O$_2$ Dependence

**MPO Inhibition by H$_2$O$_2$ Dependence.** Serial dilutions of H$_2$O$_2$ were made using 30% stock of H$_2$O$_2$. H$_2$O$_2$, mpo, adhp and sodium acetate were incubated at 25°C for 20 minutes prior to initiation. The endpoint reading for these reactions were performed using the fluorescence assay in the plate reader. The excitation and emission wavelengths were 530nm and 590nm respectively.
2.4.2 Inhibition using Sodium Azide

MPO inhibition by Sodium Azide using fluorescence assays
After determining the greatest fluorescence intensity for H₂O₂, this dilution was used in the next step of the assay. Adhp, H₂O₂, and mpo were now incubated with NaN₃ (1mM). The endpoint reading for these reactions were performed using the fluorescence assay in the plate reader. The excitation and emission wavelengths were 530nm and 590nm at 25 °C.
2.4.3 Inhibition using Benzoic Acid Hydrazide

**MPO inhibition by Benzoic Acid Hydrazide using fluorescence assays**

Adhp, H$_2$O$_2$, and mpo were now incubated with bah (1mM). The endpoint reading for these reactions were performed using the fluorescence assay in the plate reader. 530nm and 590nm were used as the excitation and emission wavelengths respectively at 25°C.
Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase

30% stock of H$_2$O$_2$, adhp, mpo, sodium acetate and inhibitors bah, tofacitinib, and ruxolitinib were incubated at 25°C for 20 minutes prior to initiation. Adhp, H$_2$O$_2$, and mpo were used in the presence of each inhibitor, bah (10mM), tofacitinib (10mM) and ruxolitinib (10mM) independently. The excitation and emission wavelengths were 530nm and 590nm respectively.
An ANOVA test was used that compared the positive control to each inhibitor. This test determined that the P value was in fact <0.0001 for bah and tofacitinib and that they were significantly different (P value <0.05). However the P value for ruxolitinib was 0.1335 and was not significantly different.
2.4.5 Ruxolitinib Free Base, Ruxolitinib Phosphate and BAH at 5mM

**Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase**

Adhp, \( \text{H}_2\text{O}_2 \), and mpo were used in the presence of each inhibitor, bah (5mM), ruxolitinib free base (5mM) and ruxolitinib phosphate (5mM) independently. The endpoint reading for these reactions were performed using the fluorescence assay in the plate reader. The excitation and emission wavelengths were 530nm and 590nm respectively.
Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase

An ANOVA test was used that compared the positive control to each inhibitor. This test determined that at this concentration BAH is significantly different from the positive control with the p value = 0.012. However ruxolitinib freebase and ruxolitinib phosphate were not significantly different with p values = 0.2286 and 0.1480 respectively.
2.4.6 MPO inhibition using ruxolitinib free base, ruxolitinib phosphate, and bah at 1mM

Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase
Adhp, H$_2$O$_2$, and mpo were used in the presence of each inhibitor, bah (1mM), ruxolitinib free base (1mM) and ruxolitinib phosphate (1mM) independently. The endpoint reading for these reactions were performed using the fluorescence assay in the plate reader. The excitation and emission wavelengths were 530nm and 590nm respectively.
Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase

An ANOVA test was used that compared the positive control to each inhibitor. This test determined that all inhibitors were significantly different. Bah was significant with a p value < 0.0001. Ruxolitinib freebase (rox fb) and ruxolitinib phosphate (rox salt) were also significant with p values of 0.0006 and 0.0001 respectively. 1 represents the positive control and 2 represents each inhibitor indicated by the color in the legend.
Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase

Adhp, H₂O₂, and mpo were incubated in the presence of each inhibitor, Bah (1mM), tofacitinib (1mM) and ruxolitinib (1mM) independently. The endpoint reading for these reactions were performed using the fluorescence assay in the plate reader. The excitation and emission endpoints were 530nm and 590nm respectively.
Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase

An ANOVA test was used that compared the positive control to each inhibitor. This test determined that the P value was in fact <0.0001 for all inhibitors and that they were significantly different (P value <0.05).
2.4.8. MPO Inhibition at 0.5mM using Rox FB, ROX Salt and BAH

Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase
Adhp, $H_2O_2$, and mpo were incubated in the presence of each inhibitor, Bah (0.5mM), ruxolitinib freebase (0.5mM) and ruxolitinib phosphate (0.5mM) independently. The endpoint reading for these reactions were performed using the fluorescence assay in the plate reader. The excitation and emission endpoints were 530nm and 590nm respectively.
Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase

An ANOVA test was used that compared the positive control to each inhibitor. The same positive control was used for each inhibitor. This test determined that at this concentration BAH is significantly different from the positive control with the p value <0.001. However ruxolitinib freebase and ruxolitinib phosphate were not significant with p values = 0.2589 and 0.0789 respectively.
2.4.9. Light scatter experiment using luminescence assay with tofacitinib and ruxolitinib in acetate buffer and phosphate buffer

Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase

Sodium acetate buffer, PBS (phosphate buffered saline) inhibitors ruxolitinib freebase, ruxolitinib phosphate, tofacitinib freebase and tofacitinib citrate were incubated at 25°C for 20 minutes prior to initiation. The blue bar represents incubation of tofacitinib citrate with 100% DMSO and the red represents the greatest concentration of tofacitinib citrate. This data will show us which concentration and buffer is best to use for the inhibitors in our fluorescence assay.
Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase

Sodium acetate buffer, PBS (phosphate buffered saline) inhibitors ruxolitinib freebase, ruxolitinib phosphate, tofacitinib freebase and tofacitinib citrate were incubated at 25°C for 20 minutes prior to initiation. The blue bar represents incubation of tofacitinib freebase with 100% DMSO and the red represents the greatest concentration of tofacitinib freebase.
Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase

Sodium acetate buffer, PBS (phosphate buffered saline) inhibitors ruxolitinib freebase, ruxolitinib phosphate, tofacitinib freebase and tofacitinib citrate were incubated at 25°C for 20 minutes prior to initiation. The blue bar represents incubation of ruxolitinib phosphate with 100% DMSO and the red represents the greatest concentration of ruxolitinib phosphate.
Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase

Sodium acetate buffer, PBS (phosphate buffered saline) inhibitors ruxolitinib freebase, ruxolitinib phosphate, tofacitinib freebase and tofacitinib citrate were incubated at 25°C for 20 minutes prior to initiation. The blue bar represents incubation of ruxolitinib freebase with 100% DMSO and the red represents the greatest concentration of ruxolitinib freebase.
Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase

Sodium acetate buffer, PBS (phosphate buffered saline) inhibitors ruxolitinib freebase, ruxolitinib phosphate, tofacitinib freebase and tofacitinib citrate were incubated at 25°C for 20 minutes prior to initiation. The blue bar represents incubation of tofacitinib citrate with 100% DMSO and the red represents the greatest concentration of tofacitinib citrate.
Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase

Sodium acetate buffer, PBS (phosphate buffered saline) inhibitors ruxolitinib freebase, ruxolitinib phosphate, tofacitinib freebase and tofacitinib citrate were incubated at 25°C for 20 minutes prior to initiation. The blue bar represents incubation of tofacitinib freebase with 100% DMSO and the red represents the greatest concentration of tofacitinib freebase.
Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase
Sodium acetate buffer, PBS (phosphate buffered saline) inhibitors ruxolitinib freebase, ruxolitinib phosphate, tofacitinib freebase and tofacitinib citrate were incubated at 25°C for 20 minutes prior to initiation. The blue bar represents incubation of ruxolitinib phosphate with 100% DMSO and the red represents the greatest concentration of ruxolitinib phosphate.
Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase

Sodium acetate buffer, PBS (phosphate buffered saline) inhibitors ruxolitinib freebase, ruxolitinib phosphate, tofacitinib freebase and tofacitinib citrate were incubated at 25°C for 20 minutes prior to initiation. The blue bar represents incubation of ruxolitinib freebase with 100% DMSO and the red represents the greatest concentration of ruxolitinib freebase.
2.4.10 Light scatter experiment using luminescence assay with tofacitinib and ruxolitinib in MEBSS buffer

Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase

MEBSS buffer, inhibitors ruxolitinib freebase, ruxolitinib phosphate, tofacitinib freebase and tofacitinib citrate were incubated at 25°C for 20 minutes prior to initiation. The endpoint reading for these reactions were performed using the luminescence assay in the plate reader. The blue bar represents incubation of tofacitinib citrate with 100% DMSO and the red represents the greatest concentration of tofacitinib citrate.
Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase

MEBSS buffer, inhibitors ruxolitinib freebase, ruxolitinib phosphate, tofacitinib freebase and tofacitinib citrate were incubated at 25°C for 20 minutes prior to initiation. The endpoint reading for these reactions were performed using the luminescence assay in the plate reader. The blue bar represents incubation of tofacitinib freebase with 100% DMSO and the red represents the greatest concentration of tofacitinib freebase.
Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase

MEBSS buffer, inhibitors ruxolitinib freebase, ruxolitinib phosphate, tofacitinib freebase and tofacitinib citrate were incubated at 25°C for 20 minutes prior to initiation. The endpoint reading for these reactions were performed using the luminescence assay in the plate reader. The blue bar represents incubation of ruxolitinib phosphate with 100% DMSO and the red represents the greatest concentration of ruxolitinib phosphate.
Screening of FDA Approved Drugs for Potential Inhibition of Myeloperoxidase

MEBSS buffer, inhibitors ruxolitinib freebase, ruxolitinib phosphate, tofacitinib freebase and tofacitinib citrate were incubated at 25°C for 20 minutes prior to initiation. The endpoint reading for these reactions were performed using the luminescence assay in the plate reader. The blue bar represents incubation of ruxolitinib free base with 100% DMSO and the red represents the greatest concentration of ruxolitinib free base.
2.5 Discussion

Our attention was focused on these two inhibitors from the start because of their similarity in structure. Even though both have FDA approvals for two different disease states, (tofacitinib for RA and ruxolitinib for Myelofibrosis) the fact there is a similarity between these two does deserve some attention. Based on the literature we know that tofacitinib inhibits mpo. Our focus was to determine the mechanism by which it does inhibit mpo.

We know that this inhibition of tofacitinib does exist so we took it a step further to determine which concentration is this inhibition the greatest. Things we took into consideration were, (1) salt formation (2) pH of the buffer used (3) solubility and (4) pka of each compound. Salts of acidic and basic drugs have in greater solubility’s than their corresponding acid or base forms[85]. Each compound, tofacitinib and ruxolitinib exist in two forms, free base and salt forms. These compounds also have acidic and basic pkas. These compounds are also both soluble in water and DMSO. The interest in salt formation has grown greatly over the past half a century and in recent years, it has become the most commonly applied technique of increasing solubility and dissolution rate in drug product development [85].

The aqueous solubility of an acidic or basic drug as a function of pH dictates the notion whether the compound will form suitable salts or not and if salts are formed, what some of their physiochemical properties. pH- solubility interrelationships also dictate what counterions would be necessary to form salts, how easily the salts may dissociate
into their free acid or base forms, what their dissolution behavior would be under different GI pH conditions and whether solubility and dissolution rate of salts would be influenced by common ions [86,87]. If the pH of a saturated solution with excess solid free base is lowered from above the pH$_{max}$ to below the pH$_{max}$, the solid phase will convert to the salt, and it is important to note that the pH will not drop below pH$_{max}$ until enough acid is added to convert the entire excess free solid base into salt [85]. The reverse is true for the conversion of a salt to the free base; no free base will precipitate out until the pH is raised above the pH$_{max}$ for that particular compound. The overall impact of counterions on salt solubility depends on the magnitude of the ksp value [85].

High throughout screening methods are now routinely applied to prepare potential salt forms of new drug candidates [88-91]. Experimental determination of drug solubility is not a single vent but is performed multiple times along the drug discovery and development process, the assays and their focus varying with the phase [92]. Among the five key physicochemical screens in early compound screening, pka, solubility, permeability, stability and lipophilicity, poor solubility tops the list of undesirable compound properties. Solubility is an easy parameter to measure but its meaning and concept of use is often different for discovery and development scientists and this can be a source of misunderstandings and controversy. In a broad sense, solubility may be defined as the amount of a substance that dissolves in a given volume of solvent at a specified temperature. [92]
Compound solubility can be defined as unbuffered, buffered, and intrinsic solubility. Unbuffered is usually in water and means that the solubility of a saturated solution of the compound at the final pH of the solution. Buffered refers to the solubility at a given pH and usually neglects the influence of salt formation with counterions. Intrinsic solubility is the solubility of the neutral form of an ionizable compound. The basic pKas for tofacitinib citrate and ruxolitinib freebase are 7.13 and 5.51, respectively. This would mean that the solubility of tofacitinib would increase with decreased pH because it is protonated at a pH 5.6 and its pKa is more basic than that of ruxolitinib. There is a reported solubility difference when the freebase or salt versions of each compound is used at pH 5.6 in the peroxidase assays. We know that ruxolitinib (free base) is soluble in water at 25-50µM and soluble in DMSO at 92µM. Ruxolitinib phosphate is soluble at 19.7µM in water and 495µM in DMSO. Tofacitinib freebase is soluble at 10-20µM in water and 320µM in DMSO. Tofacitinib citrate is soluble at 11.9µM in water and 200µM in DMSO. Tofacitinib is more protonated at a pH of 5.6 and basic than ruxolitinib. The solubility of ruxolitinib varies with pH. It is also less protonated than tofacitinib.

The trend towards lower solubility compounds was attributed to the introduction of High Throughput Screening (HTS) and of combinatorial chemistry as well as new targets that require more lipophilic molecules for efficient target affinity[92]. For highly potent, low dose compounds a lower solubility may be tolerated.
The results from the mpo fluorescence assay show us that concentration is a huge factor within this assay. Using H₂O₂ dependence the concentration of H₂O₂ did decrease over time. We used our knowledge of Beers Law (A=εlc) where ε is the molar extinction coefficient, l is the length of the cuvette and c is the concentration. We know that bah is a mpo inhibitor from previous studies discussed in the literature. At high concentrations the fluorescence intensity was low and at low concentrations the fluorescence intensity values were high. This inhibition measures the breakdown of H₂O₂. We used bah as a marker for testing tofacitinib and ruxolitinib. When the concentration was 10mM each inhibitor was compared to the positive control (H₂O₂, adhp, mpo, buffer). Using an ANOVA test, bah and tofacitinib were significantly different (<0.05) with p values <0.001. However, ruxolitinib was not significant. At a concentration of 5mM we tested ruxolitinib phosphate and ruxolitinib freebase and bah. Again bah was significantly different but both rox freebase and rox phosphate were not. The concentration was lowered further to 1mM. At 1mM all inhibitors (rox free base, rox salt, tof citrate, and bah) were extremely significant compared to the positive control. This shows us that these Janus kinase inhibitors do in fact inhibit mpo. To see if this inhibition could be seen at a lower concentration we tested the inhibitors at 0.5mM. Bah was significantly different but rox salt and rox freebase were not.

Since the majority of described HT-assays in discovery set-ups use dimethyl sulfoxide(DMSO) stock solutions for logistic reasons, compound solubility in DMSO and their long-term stability in DMSO, which can have a serious impact on screening
strategies and results [93,94,95]. We tested these inhibitors in a luminescence assay in sodium acetate buffer, phosphate buffered saline, and MEBSS buffer using a 96 well plate. Each inhibitor was incubated with DMSO and seven 1:10 dilutions were done to show at what concentration is this inhibition the greatest. The absorbance was the same for both inhibitors in MEBSS buffer. At a pH of 5.6 there is no difference or chlorloniation between both forms of the inhibitors. What the luminescence assay showed us was that at a high concentration there is precipitation and keeping the drug in solution but each compound was very soluble in 100% PBS.

2.6 Conclusion

Myeloperoxidase (MPO) is a heme protein derived from neutrophils, monocytes, and macrophages. It is well known that the defense of the organism through production of hypochlorous acid (HOCl) is the traditional role of mpo. This enzyme is activated by neutrophils and characterized by powerful pro-oxidative and proinflammatory properties.

Neutrophils are polymorphonuclear granulocytes comprised of enzyme-containing granules. Neutrophils generate from the bone marrow and account for 50%–70% of circulating leukocytes in humans. Under acute inflammation, neutrophils are the first leukocytes to respond, migrate to the site of inflammation, and kill microorganisms through phagocytosis, degranulation.
Mpo is released into the extracellular fluid in the setting of the inflammatory process. It is involved in a number of chronic inflammatory diseases including RA, psoriasis, atherosclerosis and COPD. There has been evidence that mpo does contribute to the oxidative stress seen during inflammation, which makes it a primary target of these chronic inflammatory diseases. Some mpo inhibitors such as sodium azide, 2-ABAH, and melatonin have already been identified. Testing these inhibitors in our assay gave us the direction to test the newly identified inhibitors under the same conditions.

Janus Kinase also plays a role in inflammation. Cytokines play pivotal roles in the maintenance of an appropriate immune response. Targeting cytokine receptors has been an effective means of treating immune-related disorders. In the case of cytokines, the activation of the tyrosine kinase of the Janus family, better known as JAKs, were shown to be a critical step. This family comprises four molecules namely JAK1, JAK2, JAK3 and TYK2. Recently, two small molecules that inhibit JAKs enzymatic activity have been approved for clinical use. Ruxolitinib is a JAK2/JAK1 inhibitor (with some activation JAK3 and TYK2) currently prescribed for the treatment of intermediate or high-risk myeloproliferative disorders including primary myelofibrosis post-polycythemia vera myelofibrosis and post-essential thrombocythemia myelofibrosis. Tofacitinib is a JAK3/JAK1 inhibitor (but JAK2 is also affected, albeit to a lesser extent) recently approved for the treatment of rheumatoid arthritis in patients for which methotrexate therapy was not efficacious.
In conclusion, we used a fluorescence assay to measure mpo activity using its fluorescent substrate adhp. We used a number of known mpo inhibitors, such as NaN₃, and bah before expanding to tofacitinib freebase, tofacitinib citrate, ruxolitinib free base and ruxolitinib phosphate. We showed that mpo inhibition by these Janus Kinase inhibitors was only present at a concentration of 1mM. Since both ruxolitinib and tofacitinib have been identified Janus Kinase inhibitors and with the similarity of their structures further studies should definitely be done to work on the solubility of these drugs to see if this inhibition does exist at concentrations other than 1mM. More experiments definitely need to be done to determine at which concentration is this inhibition the greatest. You can’t look at the solubility without looking at the ki, pka and degree of ionizations. The hallmark mpo fluorescence assay and the luminescence assay serve as the basis for identifying other inhibitors and also working on the solubility of the two inhibitors already identified.
Chapter 3: Visions and Reflections

I’ve always enjoyed doing research. Actually one of my committee members introduced me to research during my junior year in college. Research is something that has endless possibilities which is what intrigued me. So when I decided to attend graduate school, I was thrilled to be able to actually conduct research. When my professor gave me this project I loved it immediately. Not only was the groundwork already laid out because my former lab mate had worked on this project but it was on a topic that I was interested in. Drug discovery has always interested me so being able to work with drugs that are FDA approved was a winning situation for me. I was excited about this project from the beginning.

Research is never ending. That’s one of the things I realized while working on this project. Just because its never ending doesn’t mean accomplishments can’t be made. One of the biggest accomplishments I made with this product was my ability to become comfortable working on it. I can admit I was a little nervous working on this project at the beginning even though I liked the project. What made me nervous was the thought of looking ahead and finishing the project. How will I get to that point? Will I get to that point? These were questions that I asked myself daily when I first began working on the project. I began to fell overwhelmed. Eventually I got over it and I actually became comfortable with the project. I enjoyed it. Another big
accomplishment was making progress with the project. We are now beginning to realize the compounds could potentially have an issue with solubility. This was not previously discussed so I do feel as though this was a great accomplishment because now we know what the issues are and how we can address them.

This project has so much potential because it’s a great project. Once all of the issues are ironed out I do think this project can move forward in a great way. Testing other buffers, identifying other inhibitors and looking at the solubility are things that could be observed in the future for this project. Once we know the concentration that the mpo inhibition is best then we can elevate to murine models and even greater in humans. Before we get to the murine models I think it is worth noting that these inhibitors should also be tested in the zebra fish model. Three things I would recommend be done in this project are (1) look at the solubility of each compound involved. We know that solubility does have an impact on mpo inhibition (2) test other compounds as they could potentially be inhibitors especially if they share similarities with tof and rox or are Janus Kinase inhibitors and lastly (3) test these inhibitors in other buffers to see if this could impact this inhibition of mpo. In the literature there have been numerous studies in zebra fish especially during the developmental stages so I do think testing these inhibitors in the zebrafish model would be vital.
I have thoroughly enjoyed working on this project. I’ve learned so much about myself while working on it. I do think this project has great potential. This project definitely had some good days and bad days. One thing about research is your work ethic and willingness to do the same thing on a daily basis must be there. If you don’t have that then research isn’t for you.
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