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The Mevalonate Pathway: A Potential Therapeutic Target for JAK2-driven Myeloproliferative Neoplasms

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by

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Dedication

I dedicate this work to my sister, Elizabeth. You have inspired, encouraged, and accepted me and I thank you. You have always been there for me and it has helped me to get to the point I am today. I appreciate all of our conversations and time together. I know we share similar dreams on where the field of cancer will go. You have made my life better and I hope that this enrichment spreads. Thank you and I love you!

Dreams really do come true!

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Abstract

Myeloproliferative neoplasms (MPNs) are diseases of hematopoietic stem cell origin and are characterized by uncontrolled growth of cells of the myeloid compartment. The Philadelphia chromosome negative classical MPNs, including polycythemia vera, essential thrombocythemia, and myelofibrosis, are diseases of dysregulated JAK2 signaling. In fact, the majority of MPN patients have activating mutations in JAK2 (e.g JAK2-V617F), a tyrosine kinase that contributes to the growth and survival of myeloid cells. While MPNs were first described over sixty years ago, a significant need remains to develop therapeutic strategies for them. Inhibitors of JAK2 are currently being developed, and one inhibitor, ruxolitinib, was recently approved for certain MPN patients. Ruxolitinib has made profound impacts on improving splenomegaly and constitutional symptoms in MPN patients, but it and other JAK2 inhibitors have not significantly reduced the JAK2 mutant allele burden, and thus such inhibitors have not induced remission in these patients. The current consensus in the MPN field supports JAK inhibition for the treatment of patients, but a further understanding of MPNs and JAK2 signaling, as well as improved JAK2 inhibitors, may be necessary for treating MPN patients.

The work described in this dissertation has uncovered novel requirements for JAK2-V617F-driven signaling and transformation. We demonstrate that JAK2-V617F co-localizes with lipid rafts, cholesterol-rich microdomains within the

plasma membrane that function to serve as platforms for signaling complex formation. Signaling complex formation is a necessary component for dysregulated signaling induced by JAK2-V617F. We provide evidence that cholesterol altering-lipid raft disrupting agents attenuate JAK2-V617F-driven signaling. We also show that cholesterol-lowering statins are effective at downregulating JAK2 signaling and inducing apoptosis in JAK2-V617F-driven cell lines. Importantly, we show that statins, inhibitors of the mevalonate pathway, inhibit the growth of primary MPN cells, while the same statin doses have no effect on healthy controls. Impressively, we demonstrate that statins cooperate with multiple JAK inhibitors, including ruxolitinib, to inhibit cell growth and induce apoptosis of JAK2-V617F-driven cells.

This report establishes statin-mediated inhibition of the mevalonate pathway as a potential approach to improve MPN therapeutics. We propose future studies with statins and JAK2 inhibitors in the treatment of MPNs.

Chapter 1

Introduction

Janus Kinases

Janus kinases, or JAKs, are members of a family of proteins composed of four members in humans: JAK1, JAK2, JAK3, and Tyk2 [1-2]. JAK proteins act as important signal transducers of cytokine signaling, and have crucial roles in normal cellular physiology such as development, proliferation, survival, and immune regulation through cytokine signaling [3-5]. Knockout murine models highlight the importance of JAK proteins in normal physiology. JAK1 knockout mouse models leads to perinatal lethality with severe defects in lymphoid development [6]. JAK2 knockout mice are embryonic lethal due to defective erythropoiesis, implicating its role in erythropoietin (EPO), thrombopoietin (TPO), IL-3, and IL-5 cytokine signaling [7-8]. While JAK3 knockout mice survive, they experience severe combined immune deficiency (SCID), due to defects in the B and T cell lineages [9]. Finally, Tyk2 knockout mice survive, but have incomplete responses to cytokine stimulation [10-11]. JAK1 and JAK2 are ubiquitously expressed, while expression of JAK3 and Tyk2 is primarily seen in the lympho-hematopoietic lineages [12].

JAK Protein Structure

The JAK family members contain seven homologous domains known as the Janus homology (JH) domains (Figure 1). At the C-terminus is the JH1 domain, which contains the enzymatic tyrosine kinase region of the protein. The JH2 domain is also known as the pseudokinase domain and is similar to the kinase domain in structure. The presence of these two "kinase domains" provided the inspiration to name these proteins after Janus, the two-faced Roman God. The pseudokinase domain, however, does not function as the source of the tyrosine kinase activity of JAKs, but instead functions in an auto-regulatory fashion on the kinase domain. For example, deletion of the pseudokinase domain in JAK2 and 3 enhances kinase activity [13-14]. Up until recently, it was thought that the pseudokinase domain had no true kinase activity. Ungureanu et al. showed the pseudokinase domain is capable of phosphorylating two negative regulatory sites, Ser523 and Tyr570 [15]. Phosphorylation of Ser523 and Tyr570 results in reduced overall JAK2 kinase activity. Bandaranayake et al. in 2012 demonstrated that the pseudokinase domain was found to bind Mg-ATP in a non-canonical manner and folds as a typical eukaryotic protein kinase [16].

The JH3 domain of JAKs comprises the Src-homology-2 (SH2)-like domain and it is largely not understood how it contributes to JAK activity [17-19], although it may play a role in homodimeric interactions of JAK2 kinase [20]. JH4-7 composes the FERM (band 4.1, ezrin, radixin and moesin) domain and is responsible for interaction with the box 1 and 2 motifs on cytokine receptors.

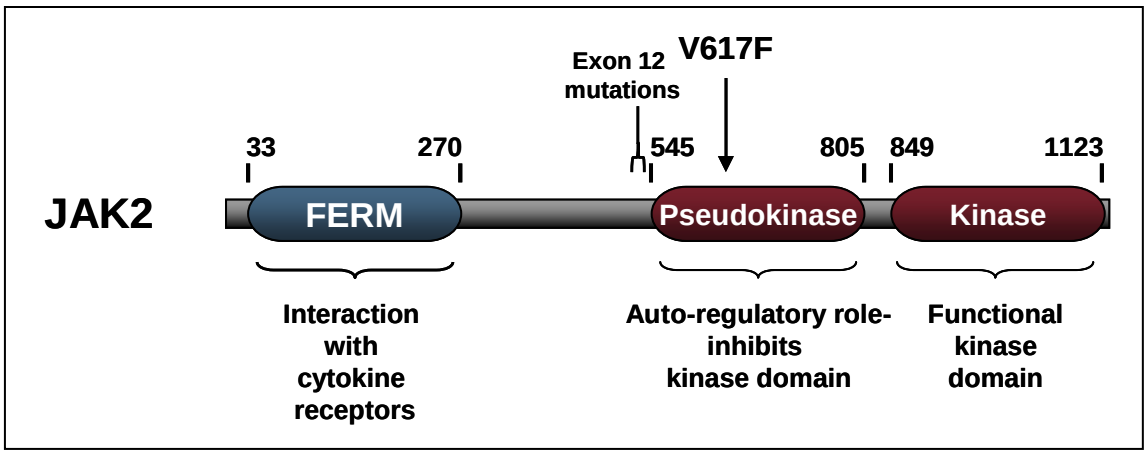


Figure 1: Schematic of JAK2 protein domains.

Cytokine Receptors and JAKs

JAKs play a crucial role in cytokine signaling by acting as signal transducers of cytokine stimulation [1]. Cytokine receptors are transmembrane receptors that harbor no intrinsic kinase activity and rely on JAKs to transduce cytokine stimulation signals from the extracellular environment, to ultimately induce changes in a cell [21]. Cytokine receptors can be divided into two general classification systems, type I and II cytokine receptors. Type I cytokine receptors, also named hemopoietin receptors, share a common amino acid motif, WSXWS located in the extracellular domain adjacent to the transmembrane domain [22]. Members of the type I receptors family include, but are not limited to: Interleukin (IL)-2-7, 9, 11, 12, 13, 15, 21, 23, 27, erythropoietin (EPO), granulocyte macrophage-colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), growth hormone, prolactin, oncostatin M (OSM), and

leukemia inhibitory factor (LIF) receptors [22]. Conversely, type II cytokine receptors include, but are not limited to: interferon (INF) alpha, beta, gamma, IL-10, 20, 22, and 28 receptors. Type II cytokine receptors share sequence similarity in the extracellular domain in that that contain tandem duplications of immunoglobulin (Ig)-like domains [22]. Cytokine receptors can bind multiple JAK family members and because all of these receptors work in dimer or oligomer complexes, can form a signaling complex with more than one type of JAK [23]. JAK1 and 2 pre-associate with cytokine receptors after final assembly of the cytokine receptor at the golgi apparatus and prior to translocation to the plasma membrane [24]. The golgi apparatus is also a site where cytokine receptors can get post-translational modifications, such as glycosylation [25]. The pre-associated receptor/JAK complex and post-translational modifications that occur at the golgi are thought to promote stability and aid in the transport of these receptors to the plasma membrane [26].

Canonical JAK/STAT Signaling

Once the receptor/JAK complex reaches the membrane, the receptor and JAK molecules maintain an inactive confirmation until stimulation by the receptor's cognate ligand (cytokine) [27-28]. Cytokine stimulation induces conformational changes in the cytokine receptor, which bring the JAKs on adjacent dimer/oligomer receptors in close proximity to transphosphorylate each other, setting off a cascade of further signaling events. Once JAK molecules are activated, they phosphorylate tyrosines on the receptor, creating docking sites for

downstream mediators, such as signal transducer and activator of transcription (STAT) molecules. STAT molecules bind to the phosphorylated receptor, which allow for activated JAKs to phosphorylate the STAT molecules. Phosphorylation of STATs allows for dimerization of STAT molecules, which allow entry of the transcription factors into the nucleus. Once nuclear, STATs bind to their target DNA elements and initiate transcription of their target genes [27-28]

Activation of JAK/STAT signaling also leads to activation of phosphotidylinositol-3'-kinase (PI3K) and AKT and ERK pathways, all of which contribute to cell proliferation, survival, blocking apoptosis, etc. [29].

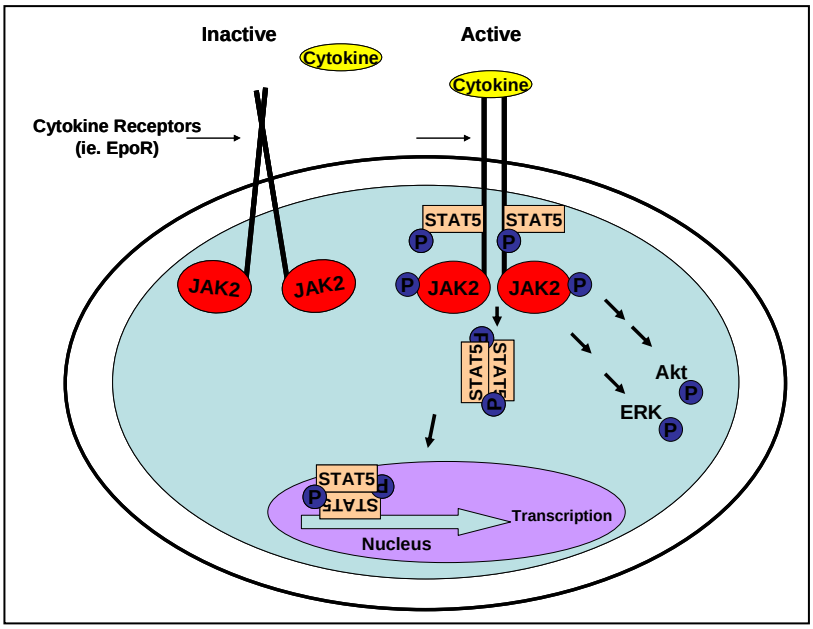


Figure 2: Canonical JAK2/STAT Signaling Pathway. Without cytokine stimulation pre-associated JAK2 and cytokine receptor complex localize to the plasma membrane in an inactive conformation. Upon cytokine stimulation, conformational changes in receptors bring JAKs in close proximity to transphosphorylate each other. Downstream mediators, such as STATs get activated and induce expression of STAT target genes. ERK and Akt pathways activation also occurs with JAK2 signaling.

Negative Regulation of Canonical JAK/STAT Signaling

There are a variety of negative regulatory mechanisms to control JAK/STAT signaling, including suppressors of cytokine signaling (SOCS) proteins, phosphatases, and protein inhibitors of activated STATs (PIAS) [30-31]. There are eight SOCS family members, SOCS1-8, that function in a classical negative feedback loop, whereby activation of JAK/STAT signaling leads to the expression of SOCS proteins. The SOCS family of proteins functions, in large part by targeting proteins for degradation through formation of an E3 ubiquitin complex [32]. All SOCS proteins contain an SH2 domain and a SOCS box, the latter functions to interact with Elongin B/C and Cullin 5, altogether completing an E3 ubiquitin complex [33-34]. However, there are additional roles associated with SOCS proteins. SOCS1 and 3 contain kinase inhibitory regions (KIRs) that bind to the kinase region of JAKs, competing with ATP and blocking kinase activity [35]. It has also been described that SOCS proteins, using their SH2 domains, bind to the same sites as downstream mediators, thus competing for binding sites and downregulating the signaling cascade [33-34]. Phosphatases including SH2 domain protein tyrosine phosphatase-2 (SHP-2), SHP-1, CD45, protein tyrosine phosphatase1B (PTP1B), and T cell PTP, among others are also important in de-activating components of the JAK/STAT pathway by removal of phosphate groups [36]. Finally, the PIAS proteins include at least four members in humans, PIAS1-4. PIAS proteins not only inhibit STAT molecules, but other proteins, namely other transcription factors [37]. PIAS proteins function to target proteins for degradation by forming complexes with small ubiquitin-related

modifer1 (SUMO1) and an E2 conjugase. However, PIAS proteins not only target transcription factors for degradation, but also act to recruit co-regulators that can block DNA binding sites, and hence transcription [37].

Non-canonical JAK Signaling

JAK signaling has been largely defined by its activity with cytokine receptors at the plasma membrane, transducing signaling from cytokine stimulation. Recently, however, JAK2 has been shown to also localize and function in the nucleus [38]. JAK1 and 2 were shown to phosphorylate tyrosine 41 of histone H3 (H3Y41), which blocks heterochromatin protein 1 alpha (HP 1α) from binding to this histone region. The complete implications of dual localization of JAKs in the cytoplasm and nucleus are not yet fully known, however, studies by Dawson et al. did provide evidence of nuclear JAK2 activity regulating the expression of the *lmo2* gene, an oncogene found in hematopoietic cells. This implicates JAK2-mediated gene expression alteration by direct histone modification. JAK2 inhibition by a small molecule inhibitor, led to a decrease in lmo2 mRNA that correlated with a decrease in phosphorylated H3Y41 and an increase in HP 1α binding at this locus, providing the first evidence of nuclear JAK2 contributing to alteration of gene expression [38]. Thus, direct roles of JAKs outside of cytoplasmic signaling are expanding to include nuclear events such as histone modification and gene expression.

JAK/STAT Pathway Mutations in Humans

Due to the role the JAK/STAT pathway has on promoting proliferation, survival, differentiation, and immunological responses, it is no surprise that this signaling cascade is aberrantly regulated in many human diseases, including cancer, hyper IgE syndrome, and severe combined immune deficiency [39]. This pathway is fundamental in blood cell production and function, and thus is found commonly mutated in hematologic malignancies including leukemia, lymphoma, multiple myeloma, and MPNs [29]. Activating mutations (including point mutations and chromosomal translocations) in JAK1, 2, and 3 are present in such malignancies [40-43]. While STAT activation is central to both wild-type and dysregulated JAK activity, STAT mutations are rare to date [29]. Some patients with large granular lymphocytic leukemia or lymphoproliferative disorders have mutated STAT-3 [44], while mutations in STAT-6 have been found in patients with primary mediastinal B-cell lymphoma [45]. Additional activating mutations that promote JAK/STAT signaling are found in receptor tyrosine kinases and cytokine receptors, including: FLT3 (found in AML) [46], KIT (AML) [47], IL-7R (acute lymphoblastic leukemia, ALL) [48-49], GCSF-R (AML, neutroplilia) [50-51], and CRLF2 (ALL) [52-53].

Conversely, inactivating JAK/STAT pathway mutations can be found in patients with myeloid malignancies. Negative regulators including: LNK (MPN/MDS) [54], SOCS1 (Hodgkin lymphoma [55], MPNs [56], mediastinal lymphoma [57], PTPN1/2 (T-cell ALL) [58], CD45 (T-cell ALL) [59], and CBL (MPNs) [60] have been reported.

Myeloproliferative Neoplasms

MPNs are a group of related hematopoietic stem cell malignancies characterized by clonal expansion of the myeloid compartment. MPNs were first described as group of related blood disorders by William Dameshek in 1951 [61]. Specifically, Dameshek grouped chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF) and he believed these disorders had their origin in defective bone marrow, with the potential to transform to myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML). It was also suggested by Dameshek that there may be an underlying genetic commonality to these phenotypically distinct MPNs [61]. With the discovery of the Philadelphia (Ph) chromosome in CML [62-63], MPNs were divided into Ph-positive (CML) and Ph-negative (PV, ET, and MF) MPNs. Subsequently, more hematologic disorders have been classified with Ph-negative MPNs, including erythroleukemia, but PV, ET, and MF remain classified together as the classical MPNs [29, 64-65]. For the purposes of this report, the term 'MPN' refers to the classical Ph-negative MPNs, PV, ET, and MF.

MPNs are characterized by an overproduction of mature myeloid cells. MPNs can be deadly on their own due to an increased risk of cardiovascular events (stroke, heart attack), progression to MF, and bone marrow failure. Importantly, MPNs can transform into AML at which point myeloid differentiation is compromised [65]. More details of these diseases will be presented in forthcoming sections.

JAK2-V617F

In 2005, five independent groups identified a common somatic mutation in the *JAK2* gene, this mutation leads to a substitution of valine to a phenylalanine at amino acid residue 617 (JAK2-V617F). The *JAK2-V617F* mutation is highly prevalent in the classical MPNs with >95% of PV, 56-60% of ET, and 50% of MF patients harboring this mutation [66-70]. With this level of prevalence, the *JAK2-V617F* mutation is now incorporated into the diagnoses of these diseases. The high prevalence of this JAK2 mutation in MPNs highly suggests it plays a causative role in these diseases.

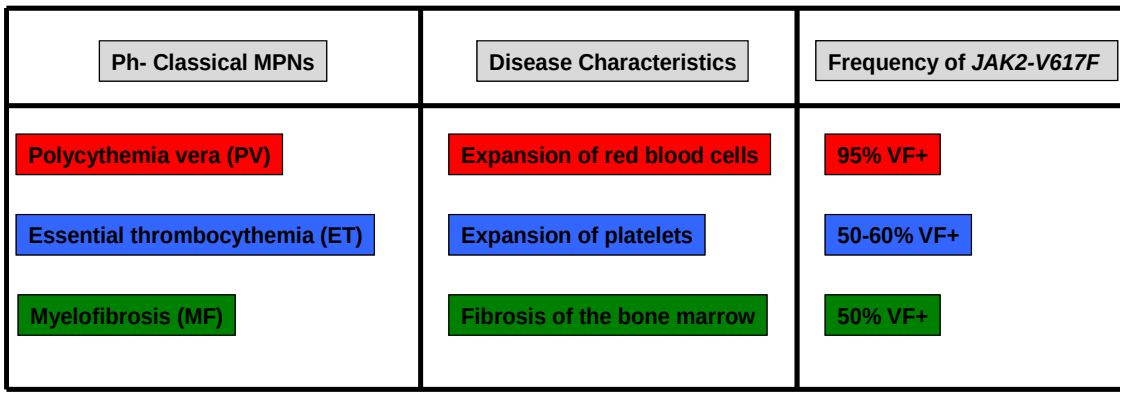


Figure 3: Characterization of Ph- classical MPNs. Table describes the major defining disease characteristics and prevalence of the JAK2-V617F mutation among Philadelphia chromosome negative (Ph-) classical MPNs, polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF).

Importantly, JAK2-V617F has been shown to be necessary and sufficient to induce an MPN phenotype in mice [71], further suggesting it plays a role in MPN development. A current hypothesis in the field is that JAK2-V617F gene

dosage contributes to the disease phenotype. That is, high JAK2-V617F expression leads to a PV phenotype, while low expression contributes to an ET phenotype, and an intermediate level contributes to MF. Interestingly, JAK2-V617F homozygosity is only found in PV and data from mouse models support this gene dosage hypothesis [71].

The V617F mutation is located in the pseudokinase domain of JAK2. Bandaranayake et al. recently resolved the crystal structure for the pseudokinase domain of both wild-type and JAK2-V617F. This work concluded there is a more rigid alpha-helix C in the N-lobe in the V617F mutant pseudokinase domain compared to the wild-type pseudokinase domain [16], providing a possible explanation for dysregulation of the JAK2-V617F mutant. It is proposed that this rigid alpha-helix C formation would allow for enhanced transphosphorylation of JAK molecules. This is presumably because of a decreased ability of the pseudokinase domain to interact with the activation loop of the kinase domain, which has been proposed by molecular dynamic simulations [16]. Additionally, the mutation of amino acid 617 to phenylalanine leads to π stacking interaction with the nearby F595, and this interaction plays a role in the elevated activation state of JAK2-V617F [72]. However, even though JAK2-V617F has elevated kinase activity due to the V617F mutation, it still requires the presence of a cytokine receptor to signal [73]. Cytokine receptors presumably provide a scaffolding function for JAK2-V617F proteins to interact and transphosphorylate each other, as well as to phosphorylate docking sites on the receptor to recruit signal transducers (Figure 4).

Other Mutations in MPNs

There is impressive evidence that JAK2-V617F contributes to MPN formation. This includes the high prevalence of the *JAK2-V617F* mutation in MPN patients, the MPN-like disease induced by JAK2-V617F in mouse models, and the structural studies of the pseudokinase domain explaining a potential mode of dysregulation of JAK2 kinase activity and subsequent signaling [65]. However, MPN patients harbor a variety of other activating and inactivating mutations. While *JAK2-V617F* is present in 95% of PV patients, it is estimated that the ~5% of PV patients that are *JAK2-V617F*-negative have mutations in exon 12 of JAK2 (K539L, etc) [74-75]. These exon 12 mutations have only been identified in PV and are thought to result in a similar manner of activation as JAK2-V617F, with loss of the auto-regulatory control on the JAK kinase domain [76]. Molecular dynamic simulations of JAK2 have suggested this may be caused in part by a shift in the salt bridge interaction of amino acid residues D620 and E621 with K539 in wild-type JAK2 to R541 in the JAK2 exon 12 mutant H538Q/K539L [77].

Activating mutations in *MPL* (myeloproliferative leukemia virus), also known as gene for the thrombopoietin receptor (TpoR) are found in 4% of ET and 10% of MF patients [78-79]. In particular, substitutions are found at amino acid residue 515, where tryptophan is substituted for leucine, lysine, or asparagine. These W515 substitutions allow for MPL to maintain an active conformation to promote JAK2 activation and signaling in the absence of cytokine stimulation [78-79].

MPN patients also display mutations in negative regulators of JAK/STAT signaling, including LNK, c-CBL, and SOCS proteins [64]. Cytokine stimulation (i.e. TPO) promotes the binding of LNK to JAK2, thereby blocking the ability of JAK2 to phosphorylate downstream mediators, such as STATs. Inactivating mutations in LNK lead to loss of function and subsequent promotion of JAK2/STAT signaling [54]. Inactivating mutations have also been identified in MPNs and myeloid malignancies in the casitas B-cell lymphoma (c-CBL) protein, an E3 ubiquitin ligase that targets components of the JAK/STAT pathway for degradation [60].

Additionally, a variety of mutations that modify epigenetic regulation are found in MPN patients, including mutations in: *TET2*, *IDH1/2*, *EZH2*, *ASXL1*, and *DNMT3A* [64]. Inactivating mutations in *TET2* are found in ~10-15% of MPN patients [80-81]. TET2 catalyzes the hydroxylation of 5-methylcytosine, and knockdown of TET2 promotes monocyte/macrophage differentiation, implicating TET2 with a suppressive effect on the differentiation of myeloid cells [82-83]. The isocitrate dehydrogenase1/2 (IDH1/2) enzymes are also mutated in ~4% of MF patients [84]. The presence of mutated IDH1/2 suggested predictive value in determining the patients that transformed to AML [84]. IDH enzymes function in catalyzing the oxidative decarboxylation of isocitrate to produce α-ketoglutarate, involved in the citric acid cycle. Rather than producing α-ketoglutarate (αKG), cancer cells with mutated IDH1/2 produce 2-hydroxyglutarate (2-HG) [85-86]. The switch over of αKG to 2-HG is to the detriment of TET2 because TET2 uses αKG as a co-activator. Without αKG, TET2 displays impaired functionality,

ultimately affecting methylation and gene expression [29]. MPN patients also display inactivating mutations in enhancer of zeste homolog 2 (*EZH2*) leading to methylation and subsequent gene expression changes. EZH2 is a histone methyltransferase that functions in conjunction with the polycomb repressor complex 2, to alter epigenetic regulation [87]. Additionally, inactivating mutations in additional sex combs like 1 (*ASXL1*), which normally binds to histones and alters chromatin remodeling by a mechanism that is not fully elucidated [88]. Mutations in DNA methyltransferase 3A (*DNMT3A*) have also been reported in MPNs. Mutated DNMT3A is thought to contribute to epigenetic alteration and subsequent gene expression alteration [89]. Multiple modes of dysregulation of the JAK2/STAT pathway have been described in MPN patients. Figure 4 depicts mechanisms of hematopoietic transformation in MPNs.

The Life of an MPN Patient

MPN patients can vary in the severity of myeloid cell expansion. While some patients have elevated levels of myeloid cells that do not require therapeutic management, others have such a high cellular burden in the periphery that management is required and life-threatening [65, 90]. The current standard of care does not change the natural history of MPNs. To manage the cellular burden, the standard of care includes chemotherapeutic agents such hydroxyurea, 2-CDA, and busulfan. Phlebotomy is also used to combat the high cellular burden in the periphery. Additional therapies for MPN patients include androgens, erythropoietin (EPO), and thalidomide to treat anemia, while

lenalidamide and interferon (IFN) pegasys may be given to patients suffering from anemia and splenomegaly [91].

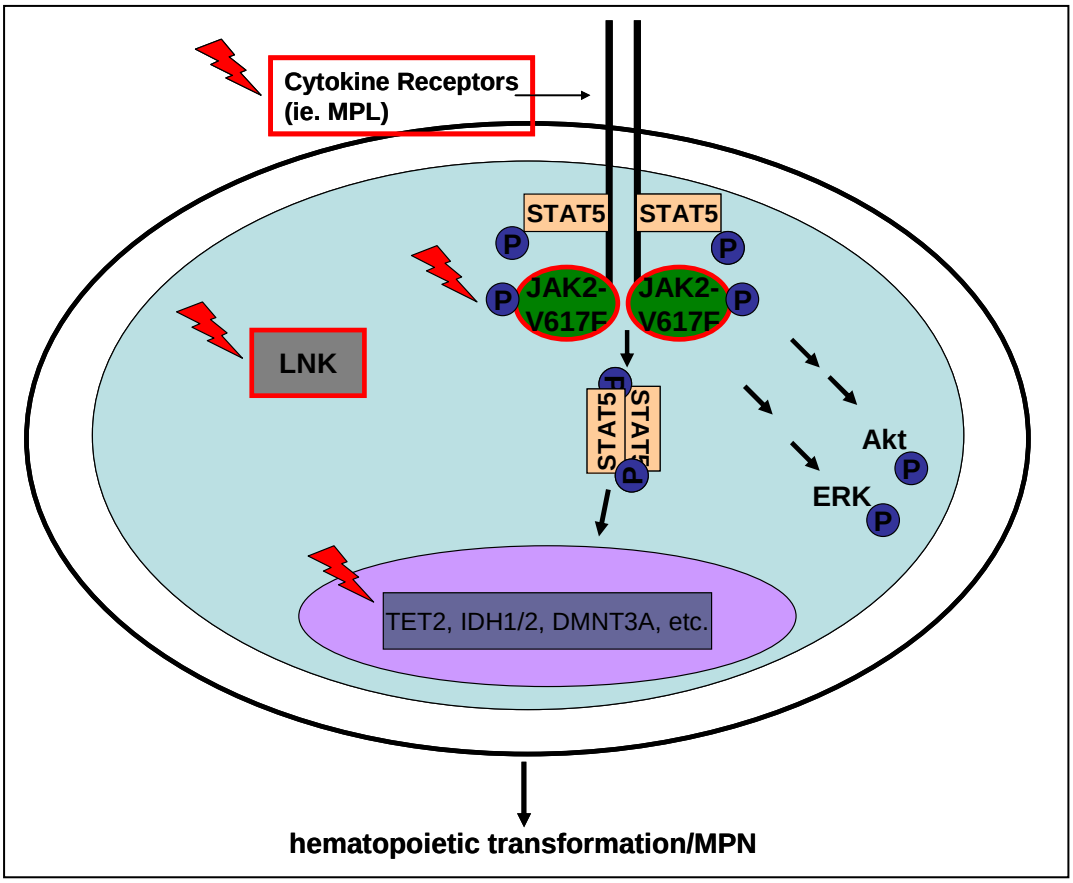


Figure 4: Mechanisms of JAK2 pathway activation in MPNs. Red lightning bolts represent mediators that are dysregulated by mutation in MPNs, these include: activating mutations in cytokine receptors (ie. MPL), activating JAK2 mutations (JAK2-V617F), inactivating mutations in the negative regulator LNK, and mutations in nuclear proteins that alter gene expression (i.e. TET2, IDH1/2, DNMT3A, etc.).

Splenomegaly, or enlargement of the spleen, occurs in MPN patients due to extramedullary hematopoiesis, or production of blood components (hematopoiesis) outside of the bone marrow.Surgery and splenic radiation can be used to control splenomegaly [91]. The only potential for a cure in MPN patients is through bone marrow transplant. However, since the average age of MPN onset is 55, bone marrow transplant is typically not an option because of the risk associated with transplant [92]. The current goal with MPN treatment is centered around preventing thrombotic events and hemorrhagic complications, leading to a lifetime of treatment for many MPN patients [90, 93]. The most serious risk for MPN patients includes transformation to acute myeloid leukemia (AML), for AML is incurable and thus leads to a dismal prognosis. MPNs can also transform into a different MPN. For example, a PV patient can transform to MF [93]. It is generally thought that cooperating mutations work with mutations that contribute to dysregulated JAK/STAT signaling. Although poorly understood, it is hypothesized that the acquisition of particular mutations is what dictates which MPN may form as well as the transformation of MPNs from one form to another and to AML [64, 93]. Further understanding these genetic and clinical dynamics is the goal of ongoing research in the MPN field.

Polycythemia vera

PV patients have a primary defect in the erythrocyte lineage, leading to expansion of the red blood cells. However, other myeloid cells may also be dysregulated and expanded in PV. Untreated PV can be life threatening [65, 94-

95]. There are approximately 22 new PV cases per 100,000 people in the U.S., which accounts for ~68,000 PV patients in the U.S [96-97]. There is a minor male predominance (1.2:1) for PV [98]. The median age of diagnosis for PV is 60 years and is rarely seen in children [98]. The overall mortality of treated PV patients is 1.6-1.7 times higher than age/sex-matched healthy individuals [99-100], with a 15 year survival rate of 65% [99]. A large international study of 1638 patients showed the leading causes of death for PV patients to include: cardiovascular complications (namely thrombosis, stroke, and hemorrhage), solid tumors, and leukemic transformation at rates of 45%, 20%, and 13% of deaths, respectively [101].

Essential Thrombocythemia

The thrombocytic, or platelet, lineage is the primary defective cell lineage in ET patients, where expansion of this lineage is seen. Similar to PV, ET patients can also display dysregulation and excess production of other myeloid lineages [102-104]. The major complications associated with ET include thrombosis and hemorrhaging because of the high platelet counts. Approximately 2% of ET patients transform to AML, while the overall 15 year survival rate for ET patients is 73% [99]. It is proposed that ET is the most prevalent Ph- MPN, with approximately 24 new ET patients per 100,000 people per year in the U.S, which accounts for ~74,000 ET patients in the U.S. [96-97].

Myelofibrosis

MF is considered one of the higher risk and heterogeneous Ph- MPNs [105]. MF patients present with a variety of clinical features including: anemia, leukocytosis or leucopenia, thrombocytosis or thrombocytopenia, constitutional symptoms, and splenomegaly [106]. Importantly, fibrosis, or scarring of the bone marrow is a key feature of MF. Depending on the number of risk factors (age >65 years; hemoglobin <10 g/dL; white blood cell count >25 X 10^9 /L; peripheral blood blasts ≥ 1%; and presence of constitutional symptoms), the survival can be predicted [107]. Patients with a low risk have zero risk factors and are expected to survive past 11 years. Patients with one risk factor are classified in the intermediate-1 group and have a median survival of 8 years. Whereas patients with two risk factors are classified in the intermediate-2 group and have a median survival of 4 years. High risk patients display ≥ 3 or more risk factors and have a median survival of 2 years [107]. MF has an incidence rate of 1.46 people in every 100,000 people in the U.S., accounting for 4,500 MF patients in the U.S. [97].

JAK Inhibitors

The BCR-ABL inhibitor, imatinib, is highly effective at blocking activity of the BCR-ABL oncoprotein, resulting in loss of the transforming signaling and thus is highly effective at killing CML cells [108-110]. The imatinib story is one of cancers therapy's best success stories with the five year survival rate being 89%. Since 95% of CML patients have the BCR-ABL translocation product, imatinib is

a highly successful treatment for CML patients [108-110]. MPNs parallel the CML scenario in many regards. A highly prevalent mutation, JAK2-V617F [64], is found in the majority of MPN patients, just as in the case of BCR-ABL in CML [108-109]. Secondly, murine models were able to recapitulate both MPNs with the expression of the single mutation, BCR-ABL recapitulated CML [111], while JAK2-V617F recapitulated MPNs [71]. Once the discovery of the JAK2-V617F mutation in MPNs was made, it was hoped the field would take a similar path as BCR-ABL-targeted imatinib therapy, with JAK inhibitor targeted therapy being highly effective for MPN patients. Thus, JAK2 inhibitors were quickly developed and moved into clinical trials.

Many JAK2 inhibitors have been developed including SAR302563, CYT387, BMS911543 [94], G6 [112], Z3 [113]. These inhibitors are effective at blocking JAK2-V617F-dependent signaling as well as JAK2-V617F-driven MPN formation in mice. Several of these inhibitors have moved into clinical trials and the JAK1/2 inhibitor, ruxolitinib, was approved by the FDA in 2011 for use in MF patients, just six years after the identification of the JAK2-V617F mutant in MPNs. Clinically, JAK inhibitors have demonstrated effectiveness in improving quality of life, reducing constitutional symptoms and splenomegaly, but have not made significant impacts on reducing the allele burden in MPN patients [94, 114]. Thus, these inhibitors have not provided remission of disease in patients. However, ruxolitinib use may improve overall survival in high-risk MF patients and thus there may be some added benefit in addition to relief of symptomology [94]. JAK inhibitors will likely play an important role in the treatment and

management of MPNs, but treatment may benefit from combination therapy in order to reduce allele burden. Even with the similarities between CML and MPNs, it is becoming clearer that MPNs are a more complex disease consisting of cooperating mutations in addition to mutations that promote JAK/STAT signaling. This is unlike BCR-ABL, which is accepted to be the causative agent in CML [115]. Others and we believe that combination therapy of JAK inhibitors with additional agents may provide an avenue to reduce allele burden and thus induce remission in MPN patients [116-117]. Further understanding the requirements for JAK2-V617F signaling toward cellular transformation may uncover additional sites for therapeutic intervention for MPNs.

Cholesterol and MPNs

Cholesterol is an essential component of the plasma membrane of mammalian cells. Due to its hydrophobic properties, cholesterol contributes to creating a barrier between the cellular contents (organelles, cytoplasm, proteins, molecules, etc.) and the extracellular environment [118]. Recently, evidence of cholesterol playing a role in MPN development in mice came to the forefront. Yvan-Charvet and colleagues found that inhibition of cholesterol efflux, through knockdown of ATP-binding cassette transporters (ABCA1 and ABCG1) resulted in an MPN phenotype in mice, implicating a role for cellular cholesterol in MPN formation. Furthermore, this group showed that high density lipoprotein (HDL), a molecule that binds cholesterol and promotes its removal from the plasma membrane, rescued the MPN phenotype in mice [119], again implicating

cholesterol in development of MPNs. HDL also inhibits hematopoietic stem cell proliferation, suggesting cholesterol plays a role in regulating blood cell growth and development. It remains unknown what role cholesterol plays in MPN development. However, since cholesterol functions in the plasma membrane in cells it is possible cholesterol-containing lipid rafts are important in this process.

Lipid Rafts

Lipid rafts are defined by the enrichment in cholesterol and sphingolipids, creating a rigid microdomain within the fluid plasma membrane [120-121]. Lipid raft biology has improved our understanding of the plasma membrane, where Singer and Nicholson initially proposed the fluid mosaic model to describe a plasma membrane. This model describes proteins and molecules moving freely and randomly in the fluid-like plasma membrane [122]. We now know there is more order to the membrane, and that order largely comes from lipid rafts. These tightly packed microdomains arise due to the interaction between the highly saturated fatty acid side-chains of sphingolipids and the polar head groups of cholesterol. In comparison to the lipids in the fluid portions of the membrane, lipid raft sphingolipid acyl chains are generally more saturated, contributing to a partition of a tightly packed microdomain within the fluid plasma membrane [120-121].

Key functions attributed to lipid rafts include providing a platform for molecular complexes with subsequent effects on signal transduction [121]. This signal transduction function can be further divided into two categories, inclusion

and exclusion. Firstly, lipid rafts can allow the inclusion of protein or molecules into the raft supporting signaling complex formation and molecular interactions. Examples of the inclusion function include signaling by EpoR [123], the platelet derived growth factor receptor (PDGF-R), and the insulin receptor [121], each of which includes the receptor and signaling mediators in lipid rafts. Secondly, lipid rafts can exclude proteins or molecules from lipid rafts, thereby sequestering components of signaling complexes. Examples of the exclusion function include the exclusion of negative regulators from lipid rafts, as in the cases of CD45, a negative regulator of JAK/STAT signaling that is excluded from lipid rafts after cytokine stimulation [124] and CD22, a negative regulator of B-cell receptor (BCR) activation that is excluded from lipid rafts during stimulation of the BCR [125]. Tied into the alteration of signal transduction function of lipid rafts, is the potential ability of lipid rafts to alter the functions of proteins due to interactions with the lipid environment within lipid rafts, whereby depending on the nature of the protein and lipid raft interactions, the protein may function differently. This is proposed for some RTKs [124]. Some viruses utilize lipid rafts to gain entry into cells [126]. Finally lipid trafficking, which aids in the transport of lipids from one region of the cell to another, is another function attributed to lipid rafts [120-121, 126].

There are two types of lipid rafts, caveolar and non-caveolar lipid rafts. Of the two types of lipid rafts, caveolae were discovered first, after flask-like invaginations were detected in the plasma membrane [127-129]. These membrane invaginations where enriched in cholesterol, sphingolipids, and a

protein called caveolin-1 (cav-1). Knockout of cav-1 in cell lines shows loss of the invaginations in the plasma membrane, suggesting a structural function for cav-1 [127]. Similar to caveolar lipid rafts, non-caveolar lipid rafts are enriched in cholesterol and sphingolipids compared to the fluid phase-plasma membrane. Also, these two forms of rafts function in the same manner, alteration of signaling transduction through inclusive or exclusive mechanisms, lipid trafficking, etc. [120-121]. The key difference between the two types of lipid rafts is the presence of cav-1, and the subsequent structural dissimilarities in plasma membrane, i.e. invaginations in the membrane.

The non-caveolar lipid rafts (for the remainder of this report, the term 'lipid rafts' refers to the non-caveolar lipid rafts) were first discovered when small regions of the plasma membrane remained intact after solublization with non-ionic detergents, hence the name detergent resistant membranes, or DRMs [120-121, 130]. In fact, it is this property that allows for experimentation and assessment of lipid rafts. Detergent solublization of the cell leads to lipid rafts remaining intact, and these non-soluble rafts can subsequently be separated from whole cell lysate. These small regions were found to be enriched in cholesterol and sphingolipids, with certain proteins being associated within these DRMs during a variety of cellular circumstances, such as activation of signal transduction [121, 130].

Lipid Rafts and JAK/STAT Signaling

Recent work by McGraw et al. demonstrated that wild-type

EpoR/JAK2/STAT5 signaling is dependent on lipid rafts (Figure 5). EPO stimulation led to an increase in lipid raft aggregates and subsequent EpoR localization within the lipid raft. Disruption of lipid rafts resulted in a downregulation of wild-type EpoR/JAK2/STAT5 signaling [123]. While JAK2-V617F-mediated signaling and transformation requires the expression of a cytokine receptor, such as EpoR, the role lipid rafts may play in dysregulated signaling by JAK2-V617F has not been investigated. This is a major focus of the studies of this dissertation.

Mevalonate Pathway

The mevalonate pathway is the key pathway responsible for cholesterol and isoprenoid biosynthesis (Figure 6). This pathway is present in most eukaryotes, archaea, and some eubacteria [131]. Overall, this pathway has important implication in cellular processes such as membrane integrity, protein prenylation, precursors for hormone production, protein anchoring, and N-linked glycosylation. The pathway begins with the conversion of acetate to acetyl-CoA, followed by the combination reaction of acetyl-CoA and acetoacetyl-CoA by 3-hydroxy-3methyl-glutaryl-CoA (HMG-CoA) synthase to form HMGCo-A. The rate limiting enzyme in the pathway, HMG-CoA reductase, catalyzes the conversion of HMGCo-A to mevalonate. Further downstream from mevalonate and after a series of reactions, is the production of isopentyl 5-diphosphate (IPP), a major precursor to downstream isoprenoid products. Subsequently, after radical coupling reactions with IPP, is the production of farnesylpyrophosphate (FPP).

FPP can also be converted to geranylpyrophosphate (GPP). The pathway branches off from the main trunk, where geranylgeranyl diphosphate synthase catalyzes the reaction that produces geranylgeranylpyrophosphate (GGPP), which gives rise to geranylgeranylation of proteins such as Ras and Rho.

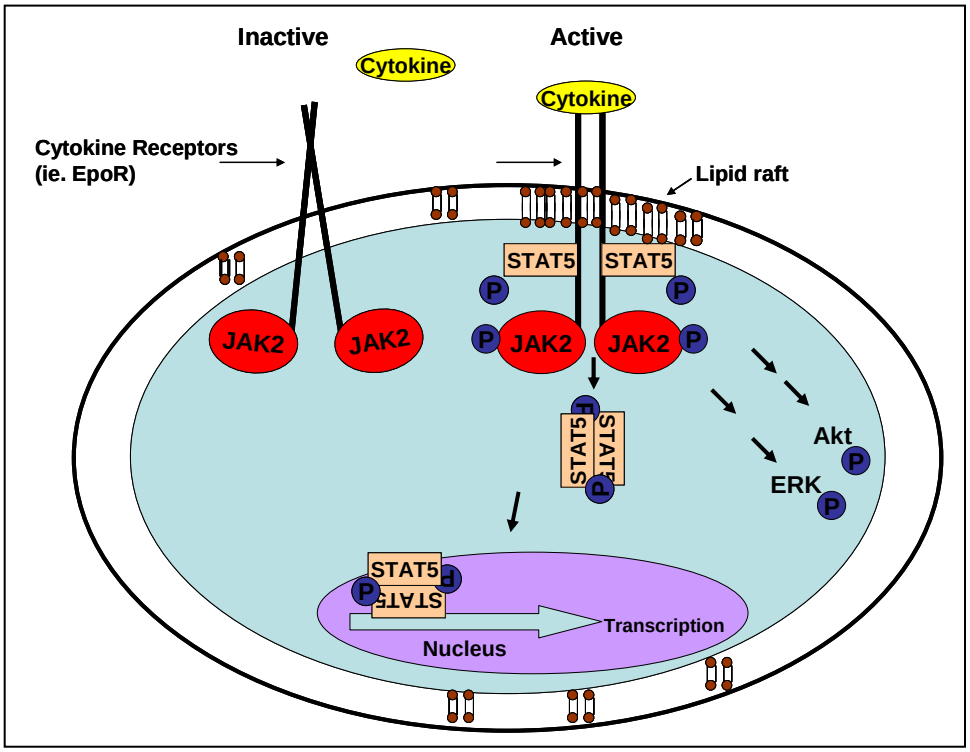


Figure 5: JAK2 signaling and lipid rafts. Without cytokine signaling, JAK2/cytokine receptor (e.g. EpoR) complexes are located outside of lipid rafts. Upon cytokine stimulation, JAK2/cytokine receptor complex localize to lipid rafts and promote JAK2 signaling cascade.

FPP can then be converted to dolichol and ubiquinone products. FPP can also be converted to squalene through a series of reactions, which can be modified to form cholesterol [131].

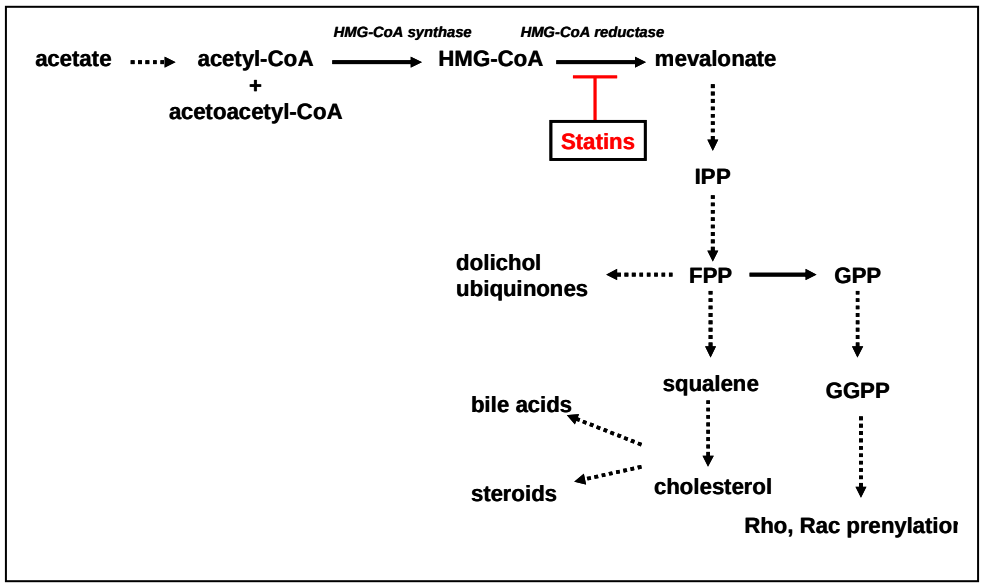


Figure 6: The Mevalonate Pathway. Dashed arrows represent multiple reactions.

The mevalonate pathway is regulated by various mechanisms [131], including the cholesterol sensing protein, sterol regulatory element-binding protein-1 (SREBP-1) which promotes expression of HMG-CoA reductase when cholesterol levels are low. High cholesterol levels promote the exposure of Lys248 of HMG-CoA reductase, leading to targeting for proteosomal degradation [132]. Negative feedback loop regulation with the mevalonate pathway end product, farnesol, has been shown to inhibit HMG-CoA reductase translation. Additional regulation is also demonstrated through phosphorylation of Ser872 on HMG-CoA reductase by AMP-activated protein kinase, effectively inhibiting enzyme activity [133]. Furthermore, cholesterol levels can also be controlled by cholesterol efflux and influx mechanisms whereby ATP-binding cassette (ABC)

transporters promote cholesterol leaving the cell, while upregulation of low density lipoprotein (LDL) receptors promote cholesterol entry into the cell [131]. Needless to say, cholesterol and the mevalonate pathway are carefully regulated by cells.

Statins

Statins are a class of drugs that inhibit HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis [134-135]. Statins, since their approval by the FDA in 1987, have been given to patients to reduce cholesterol. Statins are effective at treating hypercholesterolemia and preventing a state of hypercholesterolemia. Statins reduce the morbidity and mortality associated with cardiovascular disease [134-135]. Currently, there are seven statins on the market, with both synthetic and naturally occurring statins available [134-136]. These include, among others, simvastatin (Zocor®), atorvastatin (Lipitor®), and lovastatin (Mevacor®). All statins inhibit HMG-CoA reductase to variable degrees, and function by binding to the enzyme and thus blocking the ability of the substrate to enter the active site [137]. Currently, statins are amongst the best selling drugs in the world and recently became available in generic form in the United States. A recent estimate shows that over eight million people take statins and are generally well-tolerated [135, 138].

Statins and Cancer

Statins are the mainstay treatment to prevent and control cardiovascular diseases [138]. However, due the large numbers of patients worldwide that take statins and the large cohorts involved in the hypercholesteremia clinical trials, the effects statins impose on other aspects of health have been analyzed in a retrospective fashion. In particular, multiple reports have suggested that statins reduce the risk of some forms of cancer including: melanoma, breast, colon, prostate [139-142] and uterine cancer [143]. These and other findings promoted investigators to more directly test the effects of statins in *in vitro* and *in vivo* cancer models. Indeed, statins were found to be effective at inducing cytotoxic effects in *in vitro* cancer models including: melanoma [144], glioma [145], neuroblastoma [146], and AML cell lines [147]. Mouse models that recapitulated a variety of cancers including: ErbB2+ breast cancer [148], melanoma [149], prostate cancer [150], colorectal cancer [151], and leukemia [152] demonstrated an improvement in survival or tumor regression with statin treatment.

Statins Impact Hallmarks of Cancer

The mode in which statins elicit anti-tumor effects has been under investigation over the past decade. Multiple groups have shown statins promote a block in the tumor cell growth in prostate, gastric, AML, pancreatic, colorectal, melanoma, neuroblastoma, and mesothelioma cancer cell lines [153-154].These anti-tumor effects were in part due to the inhibition on end products of the mevalonate pathway, including, inhibition of farnesylpyrophosphate,

geranylgeranylpyrophosphate, dolichol, and cholesterol production [153]. Additionally, statins were shown to be effective at reducing proliferation induced by Ras and Rho activation, whose activities are dependent on prenylation. Furthermore, anti-tumor effects induced by statins are also explained by stabilizing cell cycle kinase inhibitors, p21 and p27 [155]. In line with blocking tumor cell growth, statins also have been shown to induce apoptosis through their ability to upregulate pro-apoptotic Bax and Bim proteins, while downregulating the anti-apoptotic Bcl-2 protein [153, 156]. Statins have also demonstrated the ability to induce the activation of caspases 3, 7, 8, and 9 [157-159].

Statins block metastatic processes by downregulating endothelial leukocyte adhesion molecule, E-selectin [160], and matrix metalloproteinase 9 (MMP 9) expression [161], and block tumor cell invasion induced by epithelial growth factor [162]. Consistent with these data, retrospective analysis of patients with prostate cancer found that there was a lower incidence of metastatic progression in patients taking low dose statins for reasons other than cancer therapeutics, than those who did not take statins [163].

Statins can also impinge on another hallmark of cancer, angiogenesis [153, 164]. Pro-angiogenic effects have been observed with low dose statin treatment of rabbits and thus statins under these conditions facilitate tumor growth. These effects are due in part by activating endothelial nitric oxide synthase and protein kinase B [165-166]. However, higher doses of statins resulted in inhibition of angiogenesis [153, 164]. High dose statin treatment can

decrease vascular endothelial growth factor secretion [167]. These data together support the concept that statin effects can vary depending on dose.

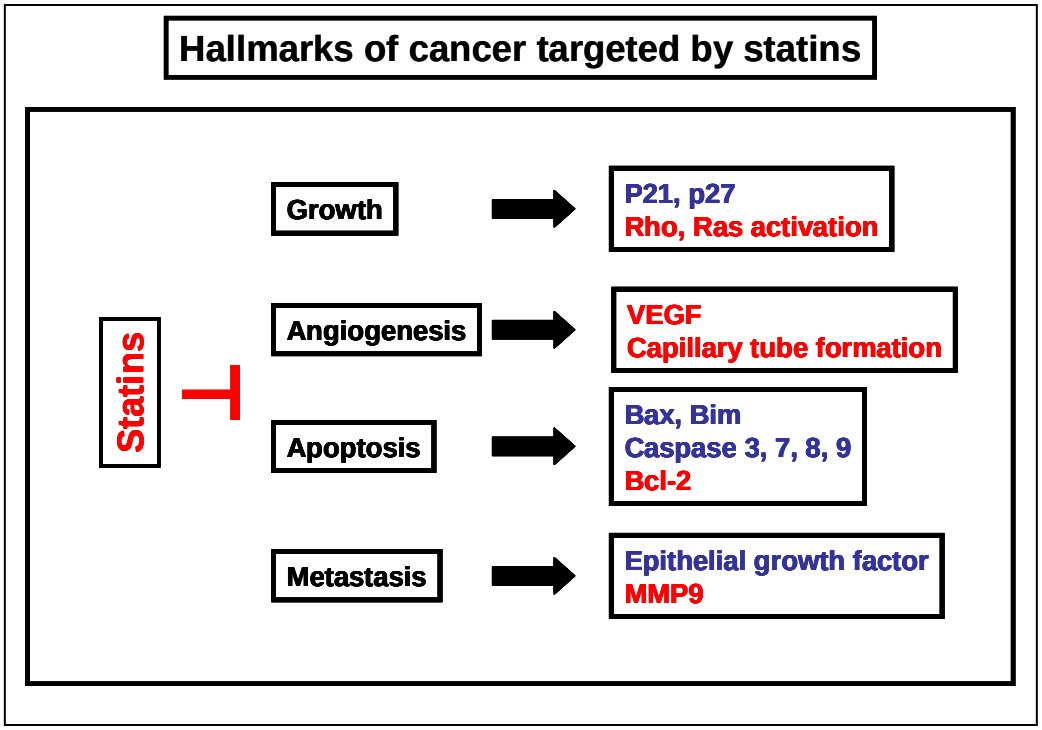


Figure 7: Statins target hallmarks of cancer. Statins block the growth capabilities of cancer by stabilizing p21 and p27, while blocking Ras and Rho-dependent growth. Statins also can inhibit angiogenesis by decreasing VEGF expression and blocking capillary tube formation. Apoptosis is also induced by statin treatment through promoting caspase 3, 7, 8, and 9 activity and inducing pro-apoptotic Bax and Bim. Additionally, statins promote apoptosis by downregulating Bcl-2 expression, an anti-apoptotic mediator. Statins are able to downregulate MMP9, while promoting epithelial growth factor, which contribute to interfering with metastatic processes. Proteins, and/or their activity, whose names are depicted in red text are negatively regulated by statins while those depicted in blue text are positively regulated by statins [153].

Clinical Trials Using Statins in Cancer Patients

Clinical trials addressing the direct effects of statins on a variety of cancers in humans have been established. Clinical trials demonstrated that statins have made positive impacts on tumor/cancer progression in humans with melanoma, colorectal cancer, breast cancer, uterine cancer, prostate cancer, and AML [153-154, 168-171]. A clinical trial testing the ability of pravastatin to improve the survival rate in patients with advanced hepatocellular carcinoma showed that patients on statins had a survival rate of 18 months, compared to control group whose median survival was 9 months [172]. Similarly, a clinical trial with non-metastatic rectal cancer showed that combined treatment with statins and neoadjuvant chemoradiation, led to 30% of the statin treated patients to have a complete response, while only 17% in the control group [173]. In addition, pro-longed administration of lovastatin in patients with squamous cell carcinoma of the head and neck resulted in 23% of the patients having stable disease compared to control group [174]. Concordantly, a small case report of a patient with acute myeloblastic leukemia showed that lovastatin treatment led to an apparent stabilization of the number of blast cells [175]. A recent report has further indicated that statins reduced the mortality associated with cancer after analyzing the entire Danish population, aged 40 years and up from 1995 to 2007 [176]. However, statins have been ineffective in clinical trials with some cancers. For example, a clinical trial testing simvastatin in chronic lymphocytic leukemia patients showed no difference compared to control group [177]. Together these data suggest that statins may have therapeutic benefit in some, but not all

cancers. Nonetheless, clinical trials testing statins in cancer patients have shown some success, supporting further investigation of statin therapy in cancer.

Statins in Combination with Other Agents

Another key aspect to statin treatment in the setting of cancer is the finding that statins have been found to enhance the effects of other established chemotherapeutic agents and cytokines [153, 178-179]. For example, lovastatin and TNFα together enhanced growth inhibition of melanoma and AML cell lines and increased survival of murine melanoma and AML models [178-179]. Cisplatin, 5-fluorouracil, and doxorubicin have also demonstrated cooperativity when combined with statins in colon cancer, melanoma, and lung cancer cell lines [180-183]. Combination treatment of lovastatin and paclitaxel exaggerated the apoptosis induction compared to single agent treatment alone in AML cell lines [184]. Interestingly, combination treatment in the case of lovastatin and doxorubicin, resulted in a reduced risk of doxorubicin-associated cardiotoxicity [182, 185], suggesting that statins, aside from inducing anti-cancer effect, may work in combination with other therapies to minimize side effects of treatment.

Chapter 2

JAK2-V617F-mediated signaling is dependent in lipid rafts and statins inhibit JAK2-V617F-dependent cell growth

Introduction

Somatic mutations in the gene encoding the JAK2 tyrosine kinase are prevalent in myeloproliferative neoplasms (MPNs) [66-70, 74], a group of hematopoietic stem cell diseases characterized in part by expansion of one or more lineages in the myeloid compartment [186]. Classical MPNs include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). Patients with PV have a defect in the erythroid lineage, leading to overproduction of red blood cells (RBCs). The main cellular defect in ET lies within the thrombocytic lineage, resulting in an overproduction of platelets. In PMF excessive blood cell formation in the bone marrow results in fibrosis of the bone marrow which can impede proper hematopoiesis [186]. A recurrent activating mutation in *JAK2*, *JAK2-V617F* is found in ~95% of PV patients and about 50% of ET and PMF patients [187]. Some *JAK2-V617F*-negative MPN patients exhibit other mutations that alter JAK2 signaling. These include exon 12 mutations of *JAK2*, mutations of cytokine receptors that signal through JAK2, and mutations of other proteins that regulate JAK2 function.

Importantly, many of these mutations can initiate an MPN-like syndrome in mouse models [187]. Collectively, these data suggest that JAK2 dysregulation contributes to MPN formation.

While JAK2 inhibitors have had significant success in recent clinical trials due to their ability to reduce constitutional symptoms and thus relieve suffering of patients, they have not readily reduced the allele burden and thus do not induce remission in patients [94, 114]. Thus, alternative therapeutic approaches that enhance neoplastic cell killing are still needed for MPN patients. Further understanding the regulation of JAK2 signaling in MPN cells may uncover additional sites of potential therapeutic intervention that may be effective at treating MPNs.

JAK2 normally functions in signal transduction initiated by cytokine receptor activation. JAK2 associates with cytokine receptors and becomes activated following cytokine receptor stimulation by ligand [39]. Cytokine binding to its receptor causes a conformational change in receptor-associated JAK2 proteins, which then trans-phosphorylate each other leading to their full activation [73]. Activated JAKs then phosphorylate the cytokine receptor, creating binding sites for downstream mediators like signal transducer and activator of transcription (STAT) molecules. STATs are then phosphorylated on tyrosines by activated JAKs [39]. Phosphorylated STATs function as transcription factors, promoting expression of genes involved in growth, survival, differentiation, etc. In the case of JAK2-V617F, the phenylalanine to valine substitution at amino acid residue 617, allows for dysregulated kinase activity through loss of an

autoregulatory function of the pseudokinase domain [188]. This mutation effectively leaves JAK2 primed for activation by circumventing the need for a conformational change of the kinase induced by cytokine receptor stimulation. However, even though JAK2-V617F does not require cytokine stimulation to be activated, a cytokine receptor is still necessary for JAK2-V617F-mediated signaling and cell transformation [73, 189]. Thus, it is thought that cytokine receptors provide a scaffolding function for JAK2-V617F-initiated signaling.

Erythropoietin receptor (EpoR) uses JAK2 to transduce signals initiated by erythropoietin (Epo) to promote RBC production [190]. We have recently shown that wild-type EpoR/JAK2 signaling requires lipid rafts [123]. Lipid rafts are microdomains of the plasma membrane that are enriched in cholesterol and sphingolipids [191]. These microdomains are more rigid than the majority of the plasma membrane and have been shown to function in membrane trafficking, cytoskeletal arrangement [120], virus entry [192-193], and cellular signaling [121]. Protein compartmentalization in membrane rafts facilitates protein interactions that regulate signal transduction activation, especially for some receptor-initiated signals at the cell surface [121].

While we have shown that wildtype EpoR/JAK2 signaling requires membrane rafts for proper signaling [123], the role of cholesterol and membrane rafts in pathologic signaling by JAK2-V617F in MPNs has never been reported, and this is what we explored in this study. We show for the first time that JAK2-V617F is localized to lipid rafts and JAK2-V617F-dependent signaling requires membrane cholesterol. By utilizing JAK2-V617F-dependent MPN model cell lines

as well as primary cells from *JAK2-V617F*-positive MPN patients, we also show that JAK2-V617F-mediated transformation is sensitive to statins, inhibitors of the cholesterol-producing mevalonate pathway. Our data showing the requirement of cholesterol for JAK2-V617F-mediated signaling and the sensitivity of MPN cells to statins suggests that statins could potentially be incorporated into a therapeutic strategy for MPNs.

Results

JAK2-V617F co-localizes with lipid rafts

HEL and SET-2 cells are widely used as MPN model cell lines to study JAK2-V617F-mediated transformation in MPNs. Each of these patient-derived cell lines expresses endogenous JAK2-V617F and requires this activated JAK2 for growth [194-195]. We first assessed if JAK2-V617F co-localized with lipid rafts in cells by utilizing immunofluorescence. We stained HEL cells, which are homozygous for JAK2-V617F [196], for GM1 ganglioside (red fluorescence), a lipid raft-associating lipid and JAK2 (green fluorescence) and used single z plane images from confocal microscopy to visualize localization. Yellow in HEL cell images represent green and red fluorescence overlap, suggesting JAK2 co-localization with lipid rafts (Figure 8A). The lipid raft disrupting agent methyl-beta cyclodextrin (MBCD) acts by binding to cholesterol and removing it from the membrane [197]. When HEL cells were treated with MBCD and stained for JAK2 and lipid rafts, single z plane images from confocal microscopy showed disruption of red staining, indicative of lipid raft disruption and thus confirming our

raft staining (Figure 8A). To reduce the possibility of false-positive staining, we used a second JAK2 antibody and obtained similar results (Figure 8B). To ensure the antibodies used in staining JAK2 in Figure 1A and 1B were specific for JAK2, we immunoblotted HEL cell lysates with the same JAK2 antibodies (Cell Signaling Technology, Inc. used in Figure 1A and Imgenex, Corp. in Figure 8B). Only a single band at the expected molecular weight for JAK2 (~125 kDa) was detected, demonstrating the JAK2 specificity of the antibodies (Figure 8C). These same two JAK2 antibodies were also used in a recent study that utilized immunofluorescence to study JAK2 sub-cellular localization in MPN cells [38].

We next employed a second method to detect the presence of JAK2-V617F protein in lipid rafts. Lipid rafts are resistant to Triton X-100 solubilization and are referred to as detergent resistant membranes (DRMs) because of these properties. They can be isolated by ultra-centrifugation based on their differential buoyant density compared to other membranes and cellular constituents [121]. We utilized an iodixanol gradient to isolate DRMs from Triton X-100 solubilized SET-2 whole cell lysate. After centrifugation, fractions were removed from the top of the gradient, resulting in lower density fractions being present in the lower numbered fractions. Equal volumes of each fraction were analyzed by dot blot analysis to identify the fractions containing the resident raft lipid marker GM1 ganglioside (Figure 9A). GM1 was detected predominantly in fraction 2, as well as in fractions 3, 5, and 6. Separation of GM1 between fractions 3 (lower buoyant density) and 5 (higher buoyant density) suggests that DRMs separated from the whole cell lysate (fractions 5 and 6), and identifies the lower buoyant

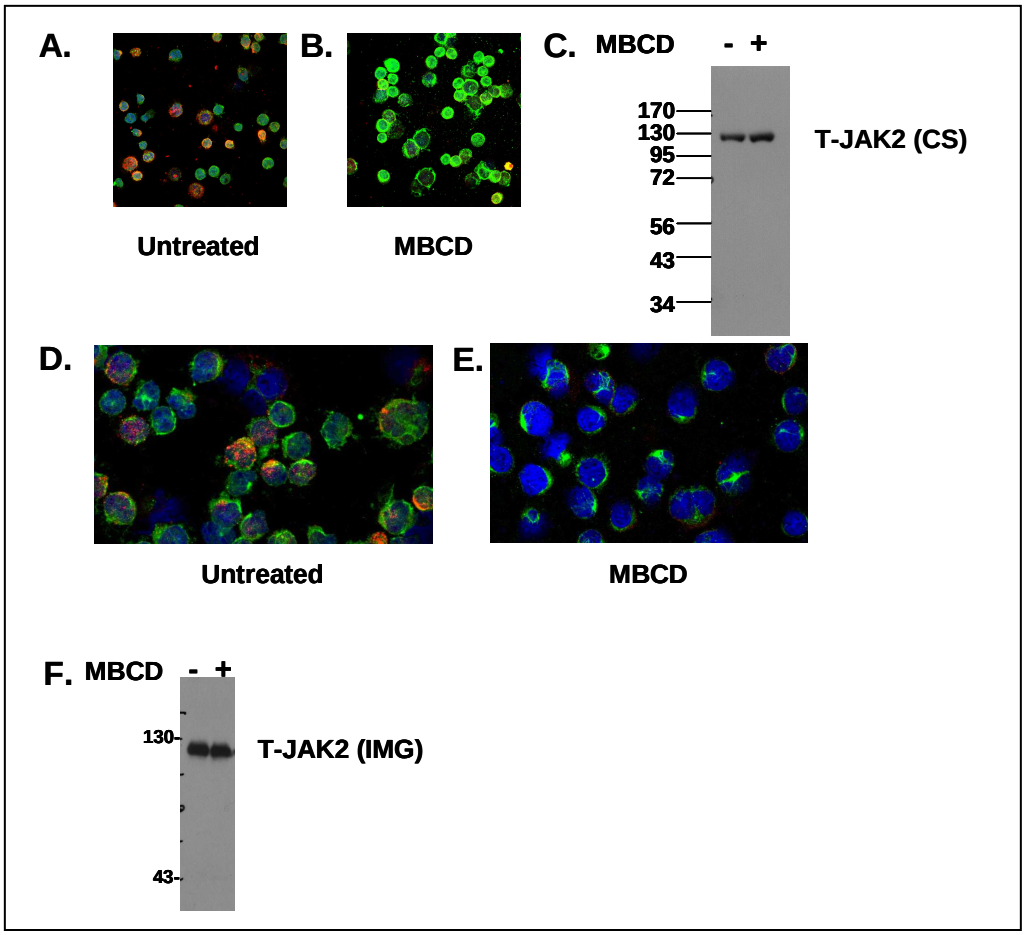


Figure 8: Mutant JAK2 co-localizes with lipid rafts in JAK2-V617F-positive cells. (A) HEL cells, untreated (left) or treated with MBCD (10 mM, 30 min, right), were stained with antibodies that recognize JAK2 (Cell Signaling Technology, CS) (green) and lipid rafts were detected by CTB, which binds to lipid raft lipid, GM1 ganglioside (red). Co-localization is demonstrated by merging green and red images, creating yellow. 4' 6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus (blue). Cells were analyzed by confocal microscopy and single z plane images are shown. (B) The experiment in (A) was repeated using a second JAK2 antibody (Imgenex, IMG). (C) Immunoblot analyses showing total JAK2 expression in HEL cells using two different antibodies, CS (left blot) versus IMG (right) blot, used in (A) and (B), respectively, are shown. Molecular weights are indicated in kDa. Immunoblots demonstrate specificity to JAK2

density fractions as raft-containing fractions (fractions 2 and 3) (Figure 9A). We then analyzed each fraction by immunoblotting for JAK2. Although the majority of JAK2 was present in the lower/cell lysate fraction (fraction 6), JAK2 was detectable in the raft fraction (fraction 2) in the untreated SET-2 cells (Figure 9B). A resident raft protein, Flotillin-1 was primarily detected in fraction 2, supporting our raft fraction designation. However, when SET-2 cells were treated with MBCD, JAK2 was no longer found in the lower buoyant density fraction 2, but solely in the higher buoyant density non-raft fractions (higher numbered fractions), suggesting that raft disruption by MBCD altered JAK2 protein localization (Figure 9C). MBCD treatment also shifted the raft marker Flotillin-1 from raft fractions to higher density buoyant fractions (higher fraction numbers), demonstrating effective disruption of lipid rafts (Figure 2C). Based on our immunofluorescence and DRM isolation data, we conclude that JAK2-V617F can be detected in lipid rafts, and the lipid raft-disrupting agent, MBCD, can abrogate this sub-cellular localization.

Lipid raft disrupting agents downregulate JAK2/STAT5 activation in JAK2-V617F-dependent cell lines

Because JAK2-V617F localization in lipid rafts was abrogated by the raft-disrupting agent, MBCD, we next investigated the effect lipid raft disrupting agents had on signaling induced by JAK2-V617F. To test this we treated the JAK2-V617F-dependent patient-derived cell lines HEL, SET-2, and Uke1 with lipid raft disrupting agents, which function by affecting membrane cholesterol.

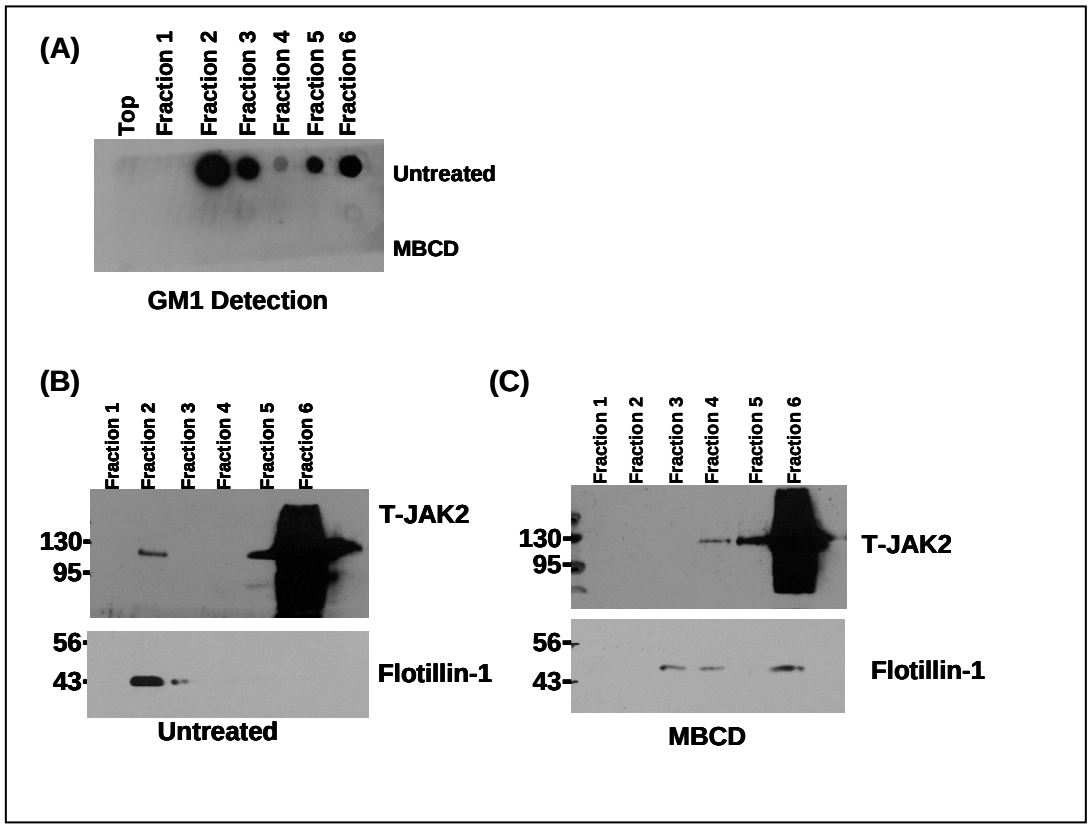


Figure 9: JAK2 is present in fractions containing detergent resistant membranes. (A) SET-2 cell lysates were analyzed by density buoyant gradient fractionation. Fractions were removed from the top of the gradient and thus lower fraction numbers correspond to lower buoyant density fractions. Each fraction was analyzed by dot blot for GM1 ganglioside, a resident raft lipid, using CTB as a probe. Separation of GM1into lower and higher buoyant density fractions suggests separation of DRMs. Highest detection of GM1 is present in fraction 2, thus designating fraction 2 as the raft fraction. (B) Immunoblot analyses of gradient fractions of untreated cells to detect JAK2 or the resident lipid raft marker, Flotillin-1. (C) Immunoblot analyses for JAK2 and Flotillin-1 as in (B), but utilizing MBCD-treated SET-2 cells. MBCD re-distributed JAK2 and Flotillin-1 to the higher density non-raft fractions. Molecular weights are indicated in kDa.

These cell lines express JAK2-V617F and JAK2 signaling, including STAT5 activation, is dependent on JAK2-V617F [194]. MBCD treatment of HEL cells

decreased P-JAK2 as shown by immunoblotting for P-JAK2 in total cell lysates (Figure 10A) and in JAK2 immunoprecipitations (Figure 10A, right panel). Activation/phosphorylation of STAT5 was effectively eliminated by MBCD treatment. Additionally, MBCD treatment resulted in a marginal decrease in P-Akt, another downstream effector of JAK2-V617F activity, in HEL cells (Figure 10A). Similar results were seen in SET-2 cells where MBCD treatment significantly decreased P-JAK2, P-STAT5, and P-Akt (Figure 10A). Likewise, MBCD treatment decreased P-STAT5 and P-AKT levels in Uke1 cells (Figure 10A). To test the effect of lipid raft disruption in a non-JAK2-V617F transformed myeloid cell line, we treated K562 cells, which display constitutive JAK2/STAT5 signaling due to the activated BCR-ABL tyrosine kinase, with MBCD. MBCD treatment did not significantly affect activation of JAK2 or STAT5 in K562 cells (Figure 10B),

Filipin complex is a lipid raft disrupting agent that functions through a different mechanism than MBCD. While MBCD removes cholesterol from the membrane, filipin complex binds to cholesterol in the membrane thereby interfering with proper lipid raft integrity [198]. Filipin complex is a weaker lipid raft disruptor than MBCD [199-200]. Filipin complex treatment of HEL cells also led to a decrease in JAK2/STAT5 activation, with a modest effect on P-JAK2 but a significant reduction of P-STAT5 (Figure 10C).

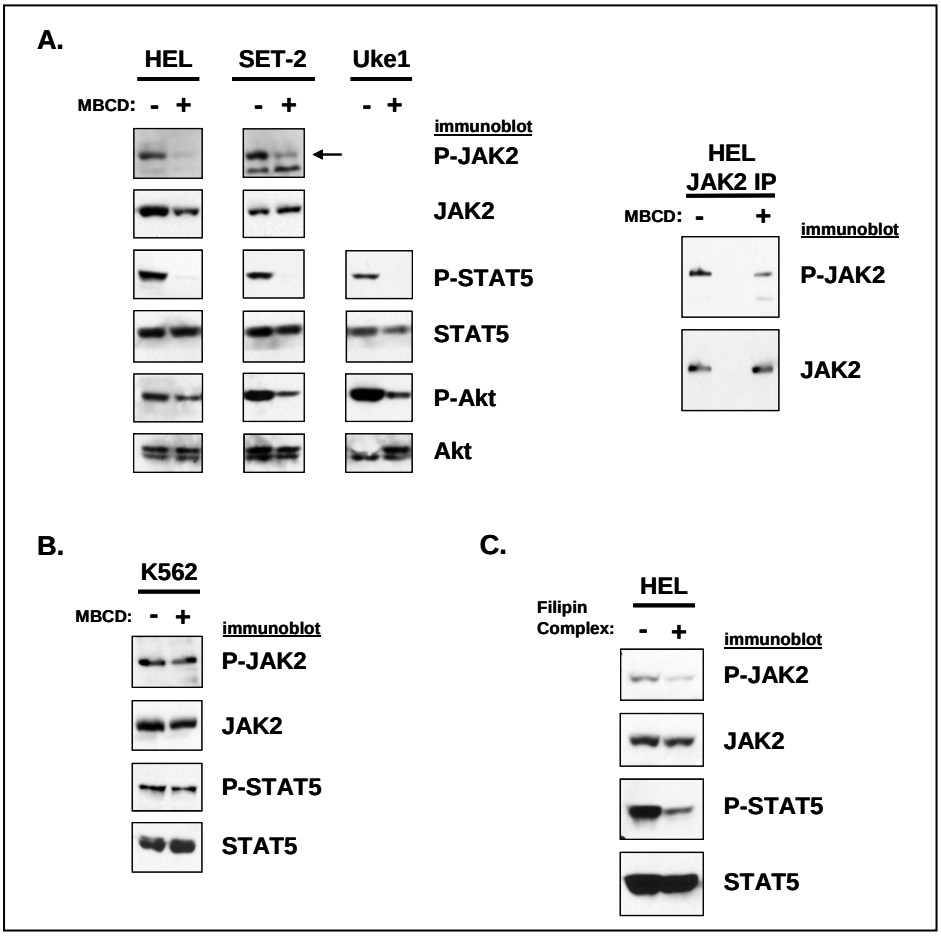


Figure 10: Lipid raft disrupting agents downregulate signaling in JAK2-V617F-dependent MPN model cell lines. (A) The JAK2-V617F-dependent cell lines HEL, SET-2, and Uke1 were left untreated (-) or treated with MBCD (10 mM) for 30 minutes (+). Lysates were analyzed by immunoblotting with antibodies that recognize phosphorylated/activated (P-) JAK2, STAT5, and Akt, as well as total JAK2, STAT5, and Akt, as indicated. Arrow indicates mobility of JAk2 (125 kDa) in SET-2 cells. (B) K562 cells, a BCR-ABL-positive CML cell line that has wild-type JAK2, but constitutive JAK2/STAT5 activation, were left untreated (-) or were treated with MBCD (+) as in (A) and analyzed by immunoblotting with antibodies that recognize P-JAK2, JAK2, P-STAT5, and STAT5, as indicated. (C) HEL cells were left untreated (-) or were treated filipin complex (1 µg/mL) for 15 minutes (+). Lysates were analyzed by immunoblotting with antibodies that recognize P-JAK2, JAK2, P-STAT5, and STAT5, as indicated.

Statins inhibit growth and viability of JAK2-V617F-dependent cells

HEL, SET-2, and Uke1 cells require JAK2-V617F signaling for growth [194-195] and our data indicates lipid raft disruption has a negative effect on JAK2-V617F-dependent signaling (Figure 10). We next wanted to disrupt JAK2-V617F localization in lipid rafts in a longer term of study in order to analyze effects on growth and survival of these JAK2-V617F-dependent cells.Statins inhibit the rate-limiting enzyme, HMG-CoA reductase, in the mevalonate pathway which leads to cholesterol biosynthesis [201] and can also be used to alter cholesterol-rich lipid rafts [202]. In addition, statins have been shown to alter the localization of cytokine receptors (e.g. EpoR) to the plasma membrane, which could also affect JAK2-dependent signaling in lipid rafts [203]. Simvastatin treatment of HEL cells, as well as SET-2 cells, led to a dose-dependent reduction in total viable cell numbers over time (Figure 11A). Similar results were seen with lovastatin and atorvastatin (data not shown). Simvastatin treatment reduced the viability of HEL cells, while the viability of K562 cells was not significantly affected (Figure 11B). Since we utilized statins to target cholesterol, we wanted to confirm cholesterol levels were indeed affected. We determined that the low dose of simvastatin we utilized in this study did indeed decrease cholesterol levels in HEL cells (Figure 11C), with 5 µM simvastatin reducing cellular cholesterol by ~34% after four days of treatment. Similarly, cholesterol reduction was observed with lovastatin and atorvastatin treatment (not shown). Finally, statin treatment inhibited the localization of JAK2-V617F to lipid rafts as determined by

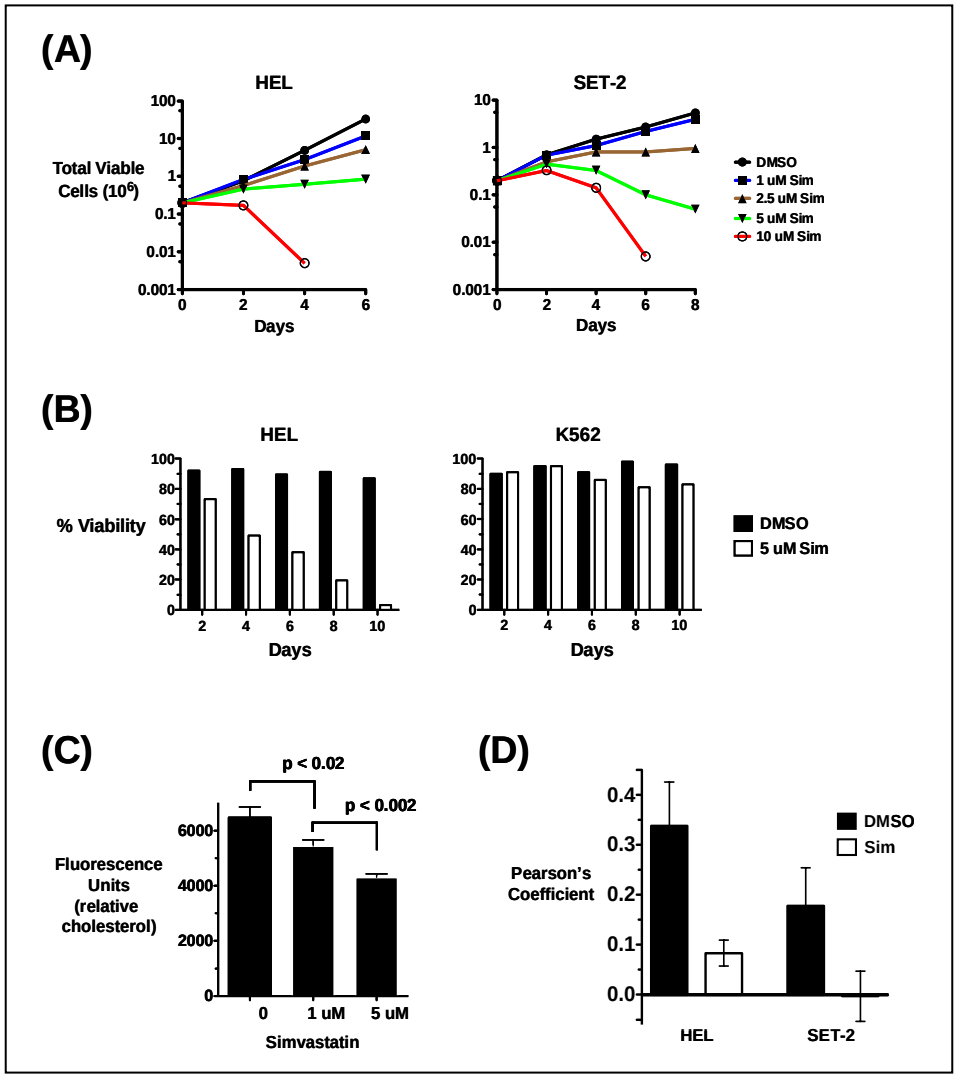


Figure 11: Simvastatin reduces JAK2-V617F-dependent cell viability and growth. (A) HEL (left graph) or SET-2 (right graph) cells were treated with 0 (0.1% dimethyl sulfoxide, DMSO) to 10 µM simvastatin (Sim). Trypan blue exclusion was used to enumerate total viable cells over time. (B) Percent viability of HEL (left graph) and K562 (right graph) cells was determined by trypan blue exclusion over time following either DMSO or 5 µM simvastatin (Sim) treatment. Data shown is representative of three independent experiments. (C) Cholesterol was measured in HEL cells after 4 d of 0 (DMSO), 1 µM, or 5 µM simvastatin treatment. Error bars indicate standard deviation and p value was determined by T-test (GraphPad Software, Inc.) This experiment is representative of three independent experiments. (D) Co-localization of JAK2-V617F and lipid rafts in HEL and SET-2 cells was performed as in Figure 1A and the Pearson's correlation analysis for co-localization was determined using Definiens Developer software. Data represents the average (with standard deviation) correlation coefficient for

immunofluorescence (Figure 11D), however it did not disrupt lipid raft formation (data not shown).

Simvastatin induces apoptosis and downregulates JAK2/STAT5 activation in JAK2-V617F-dependent cell lines

Since we observed a decrease in cell viability with statin treatment, we next assessed if statins could induce apoptosis of JAK2-V617F-dependent cell lines. Simvastatin treatment (5 µM) induced apoptosis of HEL cells as measured by annexin V staining (Figure 12A). PARP cleavage after simvastatin treatment of HEL cells for 24 and 48 hours also demonstrated simvastatin induced apoptosis in a dose and time-dependent manner, even at the very low dose of 1 µM (Figure 12B). Induction of PARP cleavage was also seen in SET-2 cells treated with simvastatin (Figure 12B). Simvastatin treatment of HEL cells reduced the activated/phosphorylated levels of JAK2 and to a less significant extent STAT5 (Figure 12C). Similar results were seen in SET-2 cells (Figure 12C). However, JAK2/STAT5 activation in K562 cells was less sensitive to simvastatin treatment than in JAK2-V617F-dependent cells (Figure 12C).

Simvastatin inhibits Primary MPN Cell Growth

Hematopoietic progenitor cells from MPN patients form erythroid colonies in methylcellulose medium lacking erythropoietin (Epo) [204]. To test the effects of statin treatment on primary MPN cells we performed colony formation assay using mononuclear cells (MNCs) from peripheral blood of two *JAK2-V617F-*

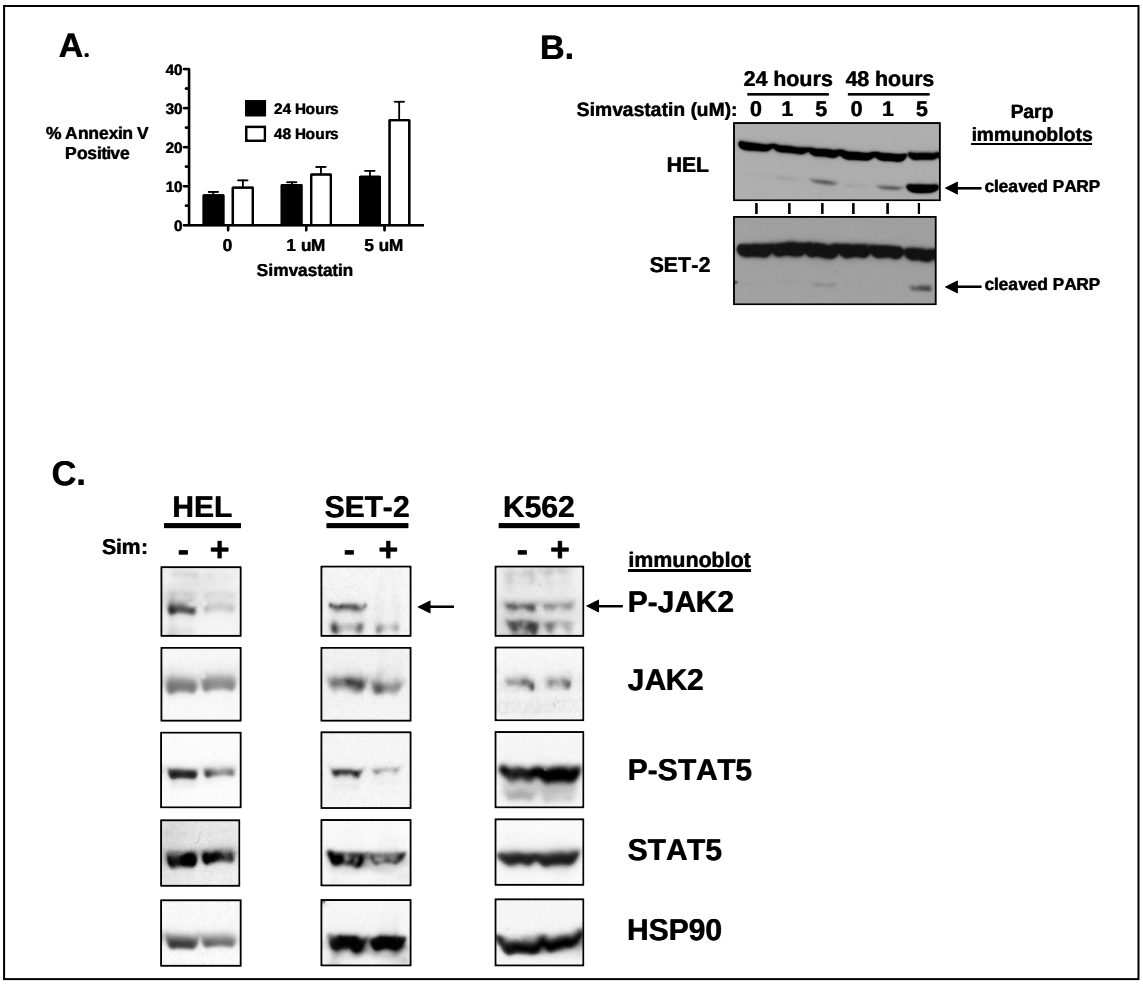


Figure 12: Simvastatin induces apoptosis and downregulates JAK2/STAT5 activation in JAK2-V617F-dependent cells. (A) HEL cells were treated 0 (DMSO), 1 µM, and 5 µM simvastatin for 24 and 48 hours and stained with Annexin V and analyzed by flow cytometry to detect Annexin V-positive cells. Error bars indicate standard deviation of triplicate samples. This experiment was performed four times with similar results. (B) Immunoblot analysis to detect PARP cleavage after 0 (DMSO), 1 µM, and 5 µM simvastatin treatment of HEL cells (top blot) and SET-2 cells (bottom blot) for 24 and 48 hours. (C) HEL, SET-2, and K562 cells were treated with 5 µM simvastatin for 4 d and cellular lysates were analyzed by immunoblotting for P-JAK2, JAK2, P-STAT5, STAT5, and Hsp90 (as additional loading control), as indicated.

positive MPN patients. Thus, *JAK2-V617F*-positive erythroid progenitors will proliferate and differentiate to form erythroid colonies in the absence of Epo. Simvastatin reduced Epo-independent erthyroid colony formation of cells from three independent MPN patients tested (Figure 13A). These patients included a *JAK2-V617F*-positive PV patient (MPN 1), a *JAK2-V617F*-positive MF patient (MPN 2), and a *JAK2-V617F*-positive post-PV/MF patient (MPN 3). Inhibition of colony formation was seen with 5 µM simvastatin. We utilized simvastatin at 2.5 µM in the experiment with MPN 3 and this lower dose inhibited colony formation to a similar extent (~75%). Similar experiments performed with cells from normal healthy controls (n=4) suggested erythroid colony formation from normal progenitor cells is unaffected by statin treatment at the same dose that shows efficacy at reducing colony formation of cells from MPN patients (Figure 13B).

Discussion

JAK inhibitor therapy was recently approved for the treatment of MF patients. JAK inhibitors have proven to be effective at improving constitutional symptoms and reducing spleen size in MPN patients. However, they do not appreciably decrease disease allele burden and thus do not induce remission in patients [94, 205]. JAK inhibitors can block the aberrant JAK2 and JAK1 signaling induced by the cytokine storm associated with MPNs, and this may be the basis for improvement in MPN patients' constitutional symptoms [94, 205]. With the inability of JAK inhibitors to decrease the allele burden in MPN patients, exploration of alternative therapeutic approaches for MPN patients continues. We

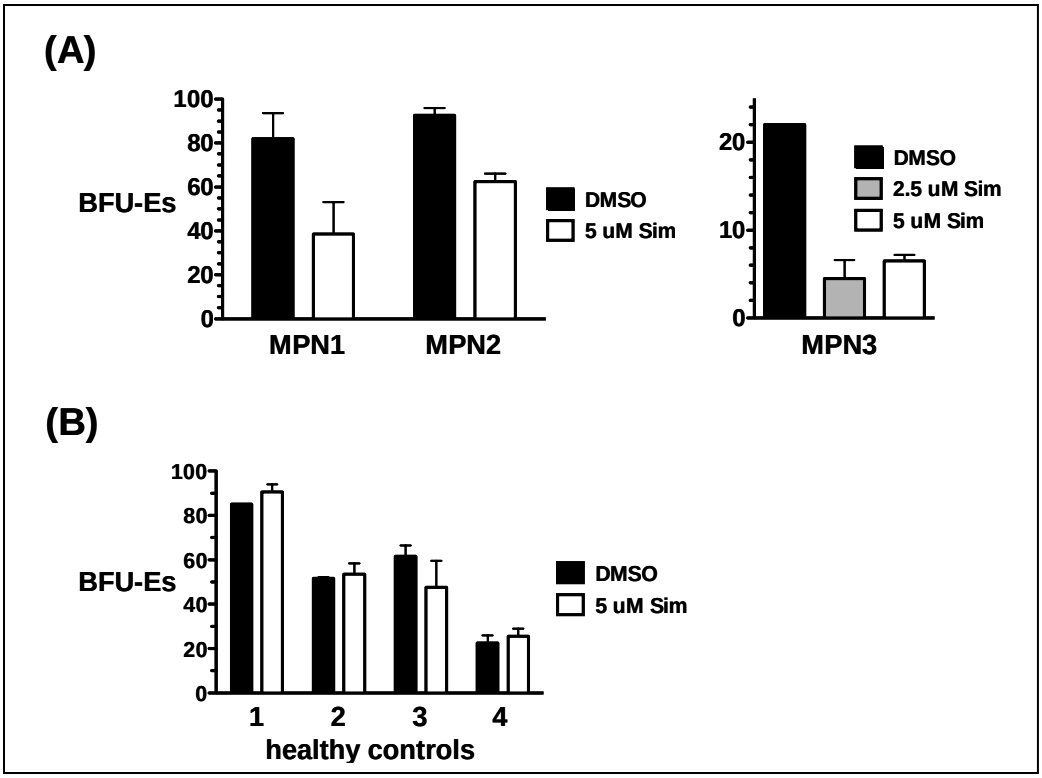


Figure 13: Simvastatin reduces erythroid colony formation of primary MPN cells. (A) Colony formation assay performed using mononuclear cells (MNCs) isolated from peripheral blood of MPN patients (n = 3). MNCs were plated in cytokine containing methylcellulose medium without Epo and Epo-independent erythroid colonies [as erythroid burst-forming units (BFU-Es)] were enumerated after 12 d of incubation. The experiment performed with either 0 (DMSO), 2.5 µM, or 5 µM simvastatin (Sim), as indicated. Data is presented as number of BFU-Es per 10^5 cells plated. MPN patients 1, 2, and 3 are a JAK2-V617F-positive PV patient, a JAK2-V617F-positive MF patient, and a JAK2-V617F-positive post-PV/MF patient, respectively. (B) The same experiment was performed with cells from healthy controls (n = 4) and with erythropoietin in the medium. All error bars represent the standard deviation of replicate plates.

initiated our studies to further our understanding of the requirements for JAK2-V617F-mediated signal transduction in an effort to uncover novel avenues for therapeutic intervention for MPNs. We recently demonstrated that EpoR/JAK2 signaling requires lipid raft formation [123] and thus wanted to determine the potential role of lipid rafts in deregulated JAK2 signaling in MPNs. While previous studies support the notion that JAK2 functions in lipid rafts [123, 206], our studies are the first to demonstrate that the MPN driver JAK2-V617F co-localizes with lipid rafts (Figures 1 and 2). Localization of this tyrosine kinase to lipid rafts is not seen in all cells, largely because not all cells exhibit raft staining (Figure 1). This may be due to the dynamic nature of lipid rafts, which is influenced by factors such as variability in raft size and half-life [207-211]. In addition, only a minor fraction of JAK2 was associated with DRMs. This is not surprising for multiple reasons. First, rafts are dynamic in nature and all cells did not display raft staining. Second, JAK2 is a cytoplasmic protein and more recently has been found in the nucleus of cells, including MPN cells [38, 212]. Third, our hypothesis is that JAK2-V617F is associated with a transmembrane receptor, such as a cytokine receptor (e.g. EpoR). Therefore, JAK2-V617F is not physically present in rafts per se, but rather associated with a protein in rafts. DRM isolation experiments utilize an overnight ultracentrifigation spin and it is likely that some JAK2 protein would not maintain its interaction with raft-associated proteins during this protocol and thus fractionate with the remainder of the JAK2, which is non-raft associated.

Using agents that disrupt cholesterol in the plasma membrane, we found that JAK2 and STAT5 activation in JAK2-V617F-dependent cells were dependent on cholesterol in the plasma membrane, while JAK2 and STAT5 activation in K562 cells, which express wildtype JAK2, were not (Figure 3). JAK2-V617F requires cytokine receptors for activation [73, 189] while wildtype JAK2 activation in K562 cells is likely induced by the BCR-ABL tyrosine kinase [213-214]. We believe that JAK2 activation by mechanisms that involve a cell surface receptor may be more sensitive to lipid raft disruption than activation of JAK2 by non-receptor mechanisms, such as BCR-ABL. The BCR-ABL induced constitutive JAK/STAT signaling may not rely on lipid rafts because the cytoplasmic BCR-ABL tyrosine kinase may activate or signal to these molecules directly [213-214]. Lipid rafts may play an integral role in the receptor scaffolding function for JAK2-V617F activation by coordinating the proper molecular complexes at the cell surface [73, 121, 189].

MPN model cell lines are also more sensitive to statin treatment than BCR-ABL positive K562 cells. We find MPN cells are sensitive to single digit micromolar statins, which is similar to certain AML cell lines, but significantly less than cells from a variety of solid tumors [215]. This may, in part, be due to the inherent driving oncogenic lesions in these cells, compared to other cancers. Statin treatment also inhibited the growth of primary MPN cells. Importantly, the growth of primary MPN cells is more sensitive to statins than cells from healthy controls (Figure 6). This is in agreement with other studies looking at the effect of statins on normal and neoplastic hematopoietic cell growth, where normal

hematopoietic cells are not sensitive to statins until high doses are achieved [215-217]. This suggests statins may be considered as a potential therapeutic agent for MPNs**,** although the effect of statins on *JAK2-V617F*-negative MPNs needs to be determined.

Although a requirement for lipid rafts in JAK2 signaling could provide a mechanistic rationale for the use of statins to inhibit MPN cells, cholesterol is not the only end product of the mevalonate pathway [169]. While we have not obtained evidence that statins inhibit lipid raft formation in our cell systems, we show that statins do appear to inhibit the localization of JAK2-V617F to lipid rafts (Figure 4D), which in effect also targets the requirement of rafts for signaling. Importantly, statins also inhibit protein prenylation by inhibiting the production of farnesyl pyrophosphate and geranygeranyl pyrophosphate, two other end products of the mevalonate pathway downstream of HMG-CoA reductase. Interestingly, it has been shown that EpoR cell surface expression requires protein geranylgeranylation [203]. It is possible that the effects of statins in MPN cells may be mediated through protein prenylation, perhaps through inhibition of a requisite cytokine receptor for JAK2-V617F-mediated signaling. In our efforts to ascertain further details regarding the mechanism of statin affects on MPN cells, we have determined that adding back geranylgeranyl pyrophosphate to cells can reduce the statin-induced loss of viability of cells, but does not significantly restore proliferation of cells (not shown). Thus, while the mechanistic details by which statins inhibit MPN cell growth are likely complex and remain to be elucidated, our data suggest that statins may be a candidate to be used as a

potential therapeutic strategy to target MPN cells. While these details will be the focus of future studies, it is important to note that statins induce MPN cell apoptosis (Figure 5A and B). This is significant since JAK2 inhibitors fail to decrease allele burden in patients, additional therapeutic approaches to complement JAK2 inhibitors are needed, especially ones that can contribute to an apoptotic response in MPN cells.

While our work is the first to directly investigate the role of lipid rafts and cholesterol in MPN cells, there is additional evidence that suggests cellular cholesterol levels could play a role in MPN cell biology. Mice deficient in cholesterol efflux transporters ABCA1 and ABCG1 display an MPN-like phenotype [119]. This suggests that an increase in cellular cholesterol in hematopoietic cells can lead to an MPN-like phenotype. In fact, Yvan-Charvet et al. [119] demonstrated that hematopoietic stem and progenitor cells from these mice displayed aberrant proliferation, and that removal of cholesterol from these cells restored a normal proliferative phenotype. These studies clearly indicate that cellular cholesterol can regulate growth control pathways of hematopoietic stem and progenitor cells, and that increasing cholesterol levels can lead to aberrant myeloproliferation. Thus, cellular cholesterol may play an important role in the development of human MPNs. Our work showing that alteration of membrane cholesterol with lipid raft disrupting agents inhibits JAK2-V617F signaling, together with Yvan-Charvet et al [119], suggests that altering cholesterol in hematopoietic stem and progenitor cells may affect cell signaling that leads to JAK2-V617F-driven myelopoiesis. Thus, altering cellular cholesterol

or inhibiting localization of JAK2-V617F to lipid rafts, perhaps through the use of statins, may be an effective approach to target the aberrant myelopoiesis associated with MPNs.

The use of statins to treat MPN patients has been previously rationally suggested [218-219]. This hypothesis is based on the antithrombotic, antiproliferative, proapoptotic, and antiangiogenic effects of statins and the role thrombohaemorrhagic complications play in MPNs. The use of statins in the treatment of MPNs has been discussed in the context of the potential role of chronic inflammation in the development of MPNs. The anti-inflammatory effects of statins may be advantageous to MPN patients as chronic inflammation may be a driving force toward clonal evolution as well as a deadly myelofibrotic state [218-219]. For example, TNFα may contribute to clonal expansion of MPN cells [220] and simvastatin lowers TNFα expression in myeloid cells in patients [221]. Also, MPN patients have an increased risk of developing both hematologic and non-hematologic secondary cancers and this may be due to the elevated inflammation associated with MPNs [91, 222]. Thus, in addition to potential direct effects of statins on MPN cells, statins may also contribute to the amelioration of disease through their anti-inflammatory effects.

In summary, we find that JAK2-V617F is associated with lipid rafts and that signaling by this constitutively activated kinase is dependent on proper lipid raft formation. Statins reduce JAK2 localization to lipid rafts, induce apoptosis of MPN cells, and inhibit colony formation of primary cells from MPN patients. Since JAK inhibitors have not had success at reducing allele burden in MPN patients,

additional therapeutic approaches are needed in order to induce remission in these patients. Our work suggests that statins might be an effective component of a therapeutic strategy for MPN patients. Additional studies are needed to investigate the potential efficacy of statins, alone and in combination with JAK inhibitors, as a potential therapeutic option for MPNs.

Materials and Methods

Immunofluorescence studies

HEL cells were treated with 10 mM MBCD (Sigma-Aldrich) for 30 minutes at 37ºC and 5% CO_2. Cells (2 X 10^6) were washed with chilled RPMI supplemented with 10% FBS, followed by a 10 minute incubation with 1 ug/mL cholera toxin B (CTB)-conjugate (Vybrant Lipid Raft Labeling Kit, Life Technologies) in chilled RPMI/10% FBS for 10 minutes at 4ºC. Cells were washed three times with chilled PBS. For experiments in which Imgenex JAK2 antibody was utilized, anti-CTB antibody (Vybrant Alexa Fluor 594 Lipid Raft Labeling kit, component B) was then performed for 10 minutes at 4ºC. Cells (2.5-5 X 10^4) were cytospun onto glass microscopes slides. Cells were then fixed using Cytofix Fixation Buffer (BD Biosciences) for 10 minutes at 37º C and washed with RT PBS. Cells were then permeabilized for 5 minutes using 2 drops of 0.5% Triton X-100 in PBS. Slides were washed using RT PBS and subsequently blocked with 2% BSA in PBS for 30 minutes at RT and washed with RT PBS. Primary antibody incubation followed, using a 1:200 dilution (in 2% BSA/PBS) for JAK2 (D2E12, Cell Signaling) or (IMG-3007, Imgenex, Corp.)

overnight (o/n) at 4º C. Slides were washed with RT PBS. Secondary antibody for JAK2 ensued using Alexa® Fluor 488 goat-anti-rabbit (Invitrogen, #411008) at 1:500 dilution (in 2% BSA/PBS) for 1 hour at RT for the JAK2 (Cell Signaling) primary, or DyLight 488 Conjugate donkey-anti goat secondary antibody (705-486147, Jackson Immunoresearch Laboratories) at a 1:500 dilution for JAK2 (Imgenex, Corp.) primary. Slides were washed with RT PBS. Mounting media (ProLong® Gold antifade reagent with DAPI, Invitrogen) was added to each slide and covered with a cover slip. Confocal microscopy with a Leica TCS SP5 AOBS laser scanning confocal microscope (Leica Microsystems, Germany) was used to image cells as previously described [123]. Definiens Developer version 1.5 (Definiens AG, Munich, Germany) was used to perform Pearson's Correlation analysis for colocalization between lipid raft and JAK2 staining on an average of 102 cells per image of four or five images per sample. Briefly, the software was used to first segment lipid raft staining areas within each cell and then perform the colocalization analysis on each pixel within these areas.

Detergent resistant membrane (DRM) isolation

SET-2 cells (12.5 X 10^6) were washed 3X with chilled PBS and lysed in 250 uL of 0.75% Triton X-100 in TNE (25 mM Tris pH 7, 150 mM EDTA, 1 mM DTT, 150 mM NaCl) plus protease and phosphatase inhibitors (1 mM sodium vanadate, 10 ug/mL leupeptin, 2 mM sodium pyrophosphate, 2 ug/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 5 minutes. Cell lysate was sonicated three times for 10 seconds each (FS60 Fisher Scientific

Sonicator, Thermo Scientific). Two hundred uL of lysate was added to 400 uL of 60% OptiPrep™ (Sigma-Aldrich) in TNE buffer, and this mixture was loaded into ultra clear ultracentrifuge tubes. Lower density OptiPrep™ solutions were loaded on top of 40% layer in decreasing order, 35%, 30%, 25%, 20% and 0%, final volume of 600 uL per layer. Samples were spun at 20,000 X g for 20 hours at 4ºC (Beckman Coulter Optima L-90K ultracentrifuge). Fractions (600 uL) were then removed from top to bottom of each gradient.

GM1 dot blots

Aliquots (5 uL) of each gradient fraction were dotted on nitrocellulose membrane, allowed to dry, and the membrane was washed with PBS. Membranes were blocked in 5% non-fat dry milk/PBS for 30 minutes at RT. GM1 detection in dot blots was performed using horse radish peroxidase conjugated cholera toxin-B (CTB) (Sigma-Aldrich, C3741) at a 1:10,000 dilution in 5% non-fat dry milk/PBS, and incubated o/n at 4ºC. Dot blots were washed 3X with 0.3% Tween-20/PBS and developed with ECL Plus (Thermo Scientific).

Immunoblotting

For DRM experiments, 50 uL of fractions were analyzed by SDS-polyacrylamide gel (SDS-PAGE). For signaling studies, 2-5 X 10^6 cells were washed with chilled PBS and lysed in lysis buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 25 mM NaF, 1% Triton X-100, plus protease and phosphatase inhibitors). Lysed cells were centrifuged at 14,500 X g for 5 minutes at 4ºC. Protein

concentration was determined using Pierce BCA reagent (Thermo Scientific) and lysates run on SDS-PAGE. Primary antibodies utilized for immunoblotting include: JAK2 (Cell Signaling, #3230), phospho- (P-) JAK2 (pY-1007/1008; Santa Cruz, sc-16566), P-STAT5 (pY694; BD Transduction, #611964), STAT5 (Santa Cruz, sc-835), Hsp90 (Santa Cruz, sc-7947), P-ERK (pT202/Y204; Cell Signaling, #4370), ERK (Santa Cruz, sc-93), P-AKT (pS473; Cell Signaling, #4060), AKT (Santa Cruz, sc-8312), and Flotillin-1 (Cell Signaling, #3253). Secondary antibodies were from Thermo Scientific. Immunoprecipitation experiments were done using JAK2 antibodies (Cell Signaling, #3230) and Protein-A agarose (Thermo Scientific). All blots were developed using West Pico Chemilluminescence, ECL Plus, or Super Signal West Femto Chemilluminescence (Thermo Scientific).

Cell growth curves

HEL or SET-2 cells were plated at 0.15 or 0.2 X 10^6 cells/mL and treated with DMSO or simvastatin (Sigma-Aldrich, #S6196). DMSO content was kept constant at 0.1% for all samples. Total cells and viability were determined by trypan blue exclusion.

Annexin V staining

HEL cells (1 X 10^6) were treated with 1 or 5 uM simvastatin for 24 and 48 hours. Cells were washed with PBS and resuspended in 100 uL 5% BSA in PBS. Fifty uL of cells were added to 50 uL 2X Annexin V Binding Buffer (BD

Pharmingen) and 11 uL staining solution (8 uL of 10ug/mL propidium iodide (BD Pharmingen) plus 3 uL Annexin V-FITC (BD Pharmingen). Cells and staining solution were incubated for 15 minutes at RT, followed by addition of 300 uL of 1X Annexin V Binding Buffer. Samples were analyzed by flow cytometry.

Cholesterol measurement

Cells (2 X 10^6) were treated with 1 or 5 uM simvastatin for 96 hours. Non-viable cells were removed by ficoll centrifugation. Cholesterol was measured using Amplex Red Cholesterol Assay kit (Life Technologies), per manufacturer's directions. Fluorescence was measured on a Synergy HT fluorometer (Biotech Instruments, Inc) using 560/590 excitation/emission settings.

Colony formation assay

Peripheral blood mononuclear cells (MNCs) were isolated by ficoll separation. Cells (0.5 - 1 X 10^5) were then plated in methylcellulose containing rhSCF, rhIL-3, and rhGM-CSF (Stem Cell Technologies, #H4534), with DMSO (0.1%) or 5 uM simvastatin. For healthy controls, Epo was included at 3 U/mL. Cells were incubated for 12 days at 37º C with 5% CO_2. Burst-forming erythroid (BFU-E) colonies were enumerated. All patients samples were obtained and utilized under informed consent through a Moffitt Cancer Center Scientific Review Committee approved protocol.

Chapter 3

Analysis of the Mechanism of Action of Statins in JAK2V617F-dependent Cells

Introduction

Statins are a class of drugs used to treat hypercholesteremia because they inhibit HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. Statins have made profound impacts on improving the morbidity and mortality of cardiovascular disease [134, 223-227]. Statin drugs are well tolerated by patients and are safe and affordable [168]. As of 2012, there were seven statins on the market and include both synthetic and naturally (fermentation) derived compounds. These statins include: atorvastatin (synthetic), fluvastatin (synthetic), pitavastatin (synthetic), rosuvastatin (synthetic), lovastatin (naturally derived), simvastatin (naturally derived), and pravastatin (naturally derived) [136]. Although the complete mechanism by which statins lower cardiovascular risk remains to be determined [153], many groups have contributed insight into the complex actions of statins in the cardiovascular setting. In general, statins have been shown to reduce cholesterol [228], inhibit protein prenylation, induce vascular remodeling through inhibition of NFkB and

matrix metalloproteinases [229], and elicit anti-inflammatory effects by modifying C-reactive protein, tumor necrosis factor-alpha, serum amyloid A, and brain natriuretic peptide levels [153, 230-232]. Importantly, statins have been shown to block growth and induce apoptosis of multiple types of cancer cells *in vitro* and *in vivo* [153], including; glioma, melanoma, prostate cancer, neuroblastoma, and leukemia [172, 217, 233-237]. Our recent studies, described in Chapter 2, demonstrated that statins have a selective cytotoxic effect in JAK2-V617F-driven MPN model cells.

Our novel findings implicated cholesterol-rich lipid rafts as having a potentially important role in the dysregulated JAK2/STAT5 signaling induced by the JAK2-V617F mutant, suggesting lipid rafts may serve as a platform for signaling complexes required for JAK/STAT signaling. Our work has shown that cholesterol-lowering statins are cytotoxic and thus growth inhibitory in JAK2-V617F-driven cell lines and primary MPN cells [238]. We had aimed to disrupt lipid rafts with cholesterol-lowering statins by targeting cholesterol biosynthesis and thus deregulating a crucial component of lipid rafts. However, HMG-CoA reductase inhibition by statins could have multiple effects in cells (i.e. inhibition of protein prenylation and, or lowering cellular cholesterol), since this enzyme is an early component in the mevalonate pathway. Thus, it is plausible that cholesterol biosynthesis may not be the only or the actual cause of the cytotoxic effects induced by statins in JAK2-V617F driven cells.

In this study, we analyzed the mechanism of action driving the cytotoxic effects induced by statins in JAK2-V617F-driven cells. We demonstrate that

multiple components of the mevalonate pathway are affected by statin treatment in MPN cells including: cholesterol levels, geranylgeranylation, and farnesylation. In addition, we show that JAK2 localization in rafts, and EpoR glycosylation are also altered upon statin treatment of MPN model cells. We have systematically assessed the mevalonate pathway end products that may contribute to the cytotoxic effects in JAK2V617F-driven cells. Our results suggest a likely requirement of multiple components of the mevalonate pathway for JAK2-V617F-driven cell growth.

Results

Assessing the contribution of cholesterol in statin-induced effects on JAK2V617F-dependent cells

We began analyzing the mechanism of action driving the cytotoxic effects of statins by assessing the cholesterol levels after treatment with cholesterol-lowering statins. Multiple statins at 1 and 5 µM doses were effective at reducing the cholesterol levels in HEL cells. A reduction in cholesterol was seen after 96 hours of treatment by simvastatin (sim), lovastatin (lov), and atorvastatin (ator) compared to the DMSO control in HEL cells (Figure 14). Specifically, we found an 18.3% (1 µM sim), 37.8% (5 µM sim), 16.8% (1 µM lov), 46.9% (5 µM lov), 46.9% (1 µM ator), and 41.2% (5 µM ator) reduction in cholesterol compared to the DMSO control in HEL cells (Figure 14).

Cholesterol add-back after statin treatment in JAK2-V617F-driven cells

We next performed add-back experiments in order to determine if cholesterol addition could restore cell growth and viability after simvastatin treatment. We supplemented our cell cultures with Synthechol, a synthetic cholesterol, and assessed viable cell numbers over time by trypan blue exclusion.

There was a minor degree of cytotoxicity associated with 0.1X and 1X cholesterol add back itself to SET-2 cells when compared to DMSO control.

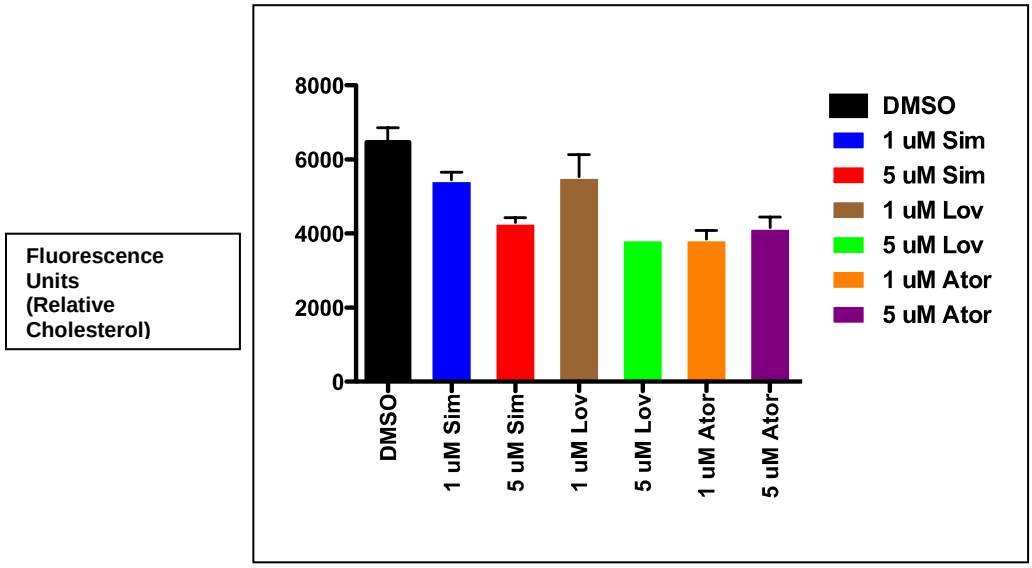


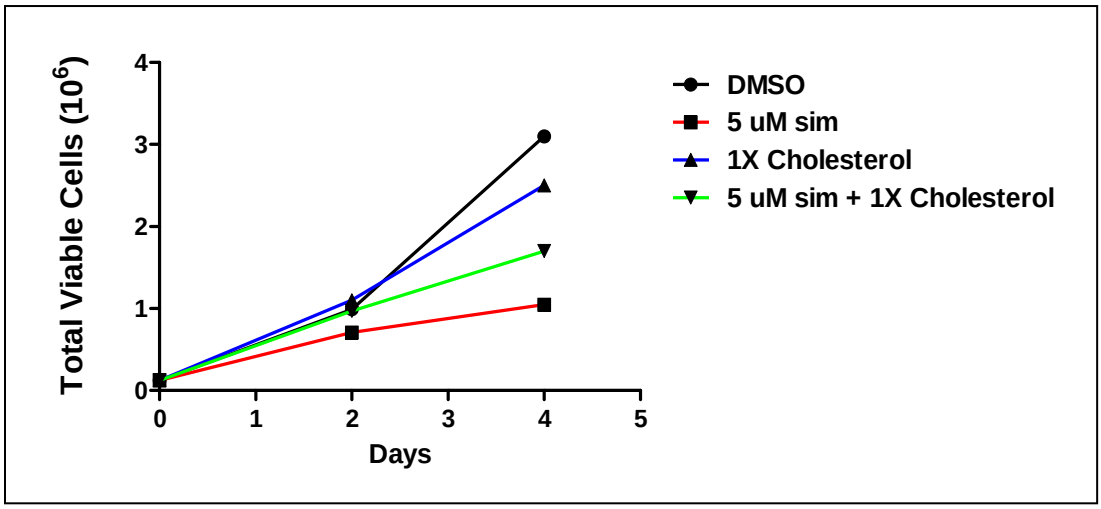
Figure 14: Statins reduce cholesterol in HEL cells. (A) A fluorescent-based cholesterol assay was used to measure cholesterol in HEL cells that were treated with 1 or 5 µM statin (simvastatin (sim), lovastatin (lov), or atorvastatin (ator)) for 96 hours. (B) The percent cholesterol reduction compared to DMSO-treated cells is graphed. Low dose statins (1 and 5 µM) were able to reduce cellular cholesterol compared to the DMSO control.

However, we found a partial restoration in total viable SET-2 cell numbers over time with the addition of cholesterol during 5 µM simvastatin treatment (Figure 15A). The % viability of SET-2 cells treated with 5 µM sim was slightly greater after two days of cholesterol treatment, while after four days of treatment the % viability was not enhanced (Figure 15B). Cholesterol addition in HEL cells was toxic so we were unable to utilize these cells for these analyses (data not shown).

Evaluating the effects of statins on lipid rafts and JAK2 localization in lipid rafts

Our previous studies showed that lipid raft disrupting agents such as MBCD (methyl-beta-cyclodextrin) and filipin complex, downregulated JAK2/STAT5 signaling in cell lines that were dependent on the transforming JAK2-V617F mutant (Chapter 2) [238]. To address the potential of statins to disrupt lipid rafts in JAK2-driven cells, we evaluated the effect of atorvastatin on lipid raft integrity in SET-2 cells. Detergent resistant membranes (DRMs) were isolated using a gradient-ultracentrifugation technique. DRMs were assessed by dot blot analysis, detecting GM1, a lipid raft-associating lipid using cholera toxin-HRP as a probe. Separation between the upper and lower fractions suggests that the lower buoyant density lipid rafts separated from the higher buoyant density whole cell lysate.

A.



B.

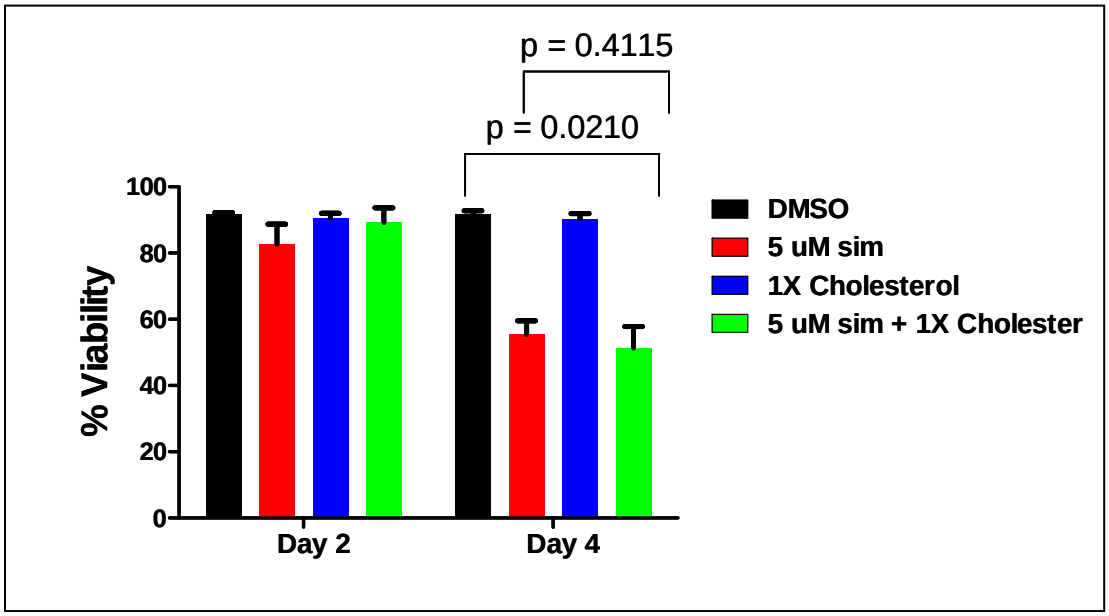


Figure 15: Cholesterol add back partially restores the cytotoxic effects induced by simvastatin in SET-cells. (A) SET-2 cells were treated with 0 (DMSO), 5 µM simvastatin (sim), 1X cholesterol, or 5 µM sim with 1X cholesterol and counted every two days by trypan blue exclusion. Addition of 5 µM sim and 1X cholesterol resulted in a partial increase in viable cell numbers compared to the 5 µM sim treatment alone. (B) The viability of SET-2 cells was assessed after 5 µM sim and cholesterol addition and compared to DMSO control and 5 µM sim alone. Cholesterol addition with 5 µM sim slightly enhanced the viability at Day 2 compared to the 5 uM sim alone sample. At day 4, no restoration in viability was seen under the same conditions. Experiments were replicated three times. Error bars indicate standard deviation and p value was calculated using T-test (GraphPad Prism, Inc.).

We found that 5 µM atorvastatin did not disrupt lipid rafts as shown by the detection of GM1 in the low buoyant density raft fraction (primarily fraction 2), which is separated by fractions 3 and 4, making fractions 5 and 6 the non-raft fractions (Figure 16A). In support of the dot blot data, we analyzed Flotillin-1, a resident raft protein, by western blot and detected Flotillin-1 in the lower buoyant density fractions (2 and 3) in the 5 µM ator treated SET-2 cells, similar to the DMSO control (Figure 16B), suggesting there was no noticeable disruption in lipid rafts (Figure 4B). Nevertheless, when assessing the expression of JAK2 across the fractions, we did find less JAK2 in the DRM (fraction 2 and 3) in the 1 and 5 µM ator treated SET-2 cells compared to the DMSO control (Figure 16B). This suggests JAK2 protein localization within DRMs had been affected by statin treatment. Similar results were seen with 5 µM simvastatin in SET-2 cells (data not shown). We previously reported that statins reduced the localization of JAK2-V617F and lipid rafts as shown by immunofluorescence (Chapter 2) [238], but did not significantly reduce lipid raft aggregates, which is in support of our raft fractionation data showing atorvastatin did not fully disrupt lipid rafts but lowered JAK2-V617F detection in the lipid raft fractions (Figure 12).

Mevalonate restores the cytotoxic effect of statins in JAK2-V617F-driven cells

To address the multiple end products, aside from cholesterol, in the mevalonate pathway, we performed add back experiments with intermediates of the mevalonate pathway to simvastatin treated JAK2-V617F-driven cell lines and

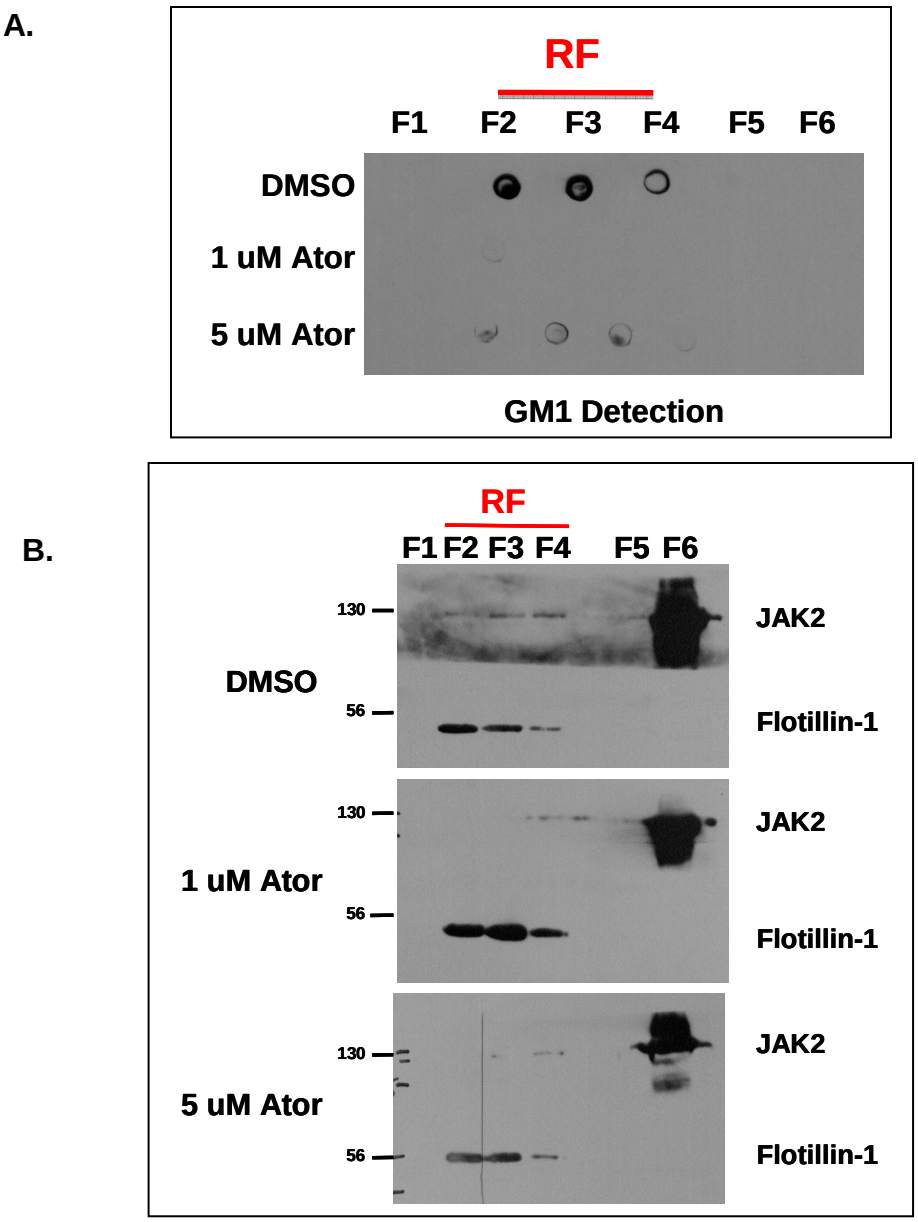


Figure 16: Atorvastatin alters JAK2 localization in detergent resistant membranes. (A) SET-2 cells were treated with 0 (DMSO), 1, or 5 µM atorvastatin (ator) for 96 hours and were analyzed similarly to the experiment in Figure 8A by density gradient separation, where lower buoyant density raft fractions were separated from higher buoyant density non-raft fractions. Dot blot analysis demonstrates GM1 detection in fraction 2 in DMSO control, with GM1 detected in fraction 2 in the 5 µM ator sample, suggesting lipid rafts were not fully disrupted. (B) Western blot analysis from gradient fractionation lysates was performed similarly to experiment in Figure 8B. Total JAK2 and Flotillin-1 were blotted, as indicated. Flotillin-1, a resident raft marker, is detected in lower buoyant density raft fractions (2-4), suggesting lipid rafts were not fully disrupted. However, reduced JAK2 is detected in the 5 µM ator sample, suggesting alteration of JAK2 localization in lipid rafts.

assessed the effects on the total viable cell numbers over time. We started with an early intermediate in the mevalonate pathway, mevalonate, which is the product of HMG-CoA reductase reducing HMG-CoA (refer to Figure 6). Mevalonate (100 µM) restored the total viable cell number of HEL cells treated with 1 and 5 µM sim (Figure 17).

Assessing the role of farnesylation in statin action in targeting JAK2-V617F-driven cells

Further downstream the pathway is the end product, farnesyl. The first method we employed to detect potential inhibition of farnesylation involved probing samples for cleavage of HDJ-2, a protein that is exclusively farnesylated. Western blot analyses of HDJ-2 can distinguish the farnesylated form (lower molecular weight, ~44 kDa) and the unfarnesylated form (~49 kDa) of HDJ2. Inhibition of farnesylation results in an increase in the presence of the upper band and thus results in a double band by western blot due to detection of the unfarnesylated HDJ-2 protein. Higher doses (5 µM or higher) of sim or lov displayed a minor induction of the unfarnesylated HDJ-2 protein band, while both 1 and 5 µM ator displayed a single band for HDJ-2, suggesting little to no inhibition of farnesylation (Figure 18A). As a positive control for farnesylation inhibition, we treated HEL cells with 1 µM FTI-2153 for 48 hours and assessed HDJ-2 by western blot. The HDJ-2 doublet validated that farnesylation was inhibited (Figure 18A).

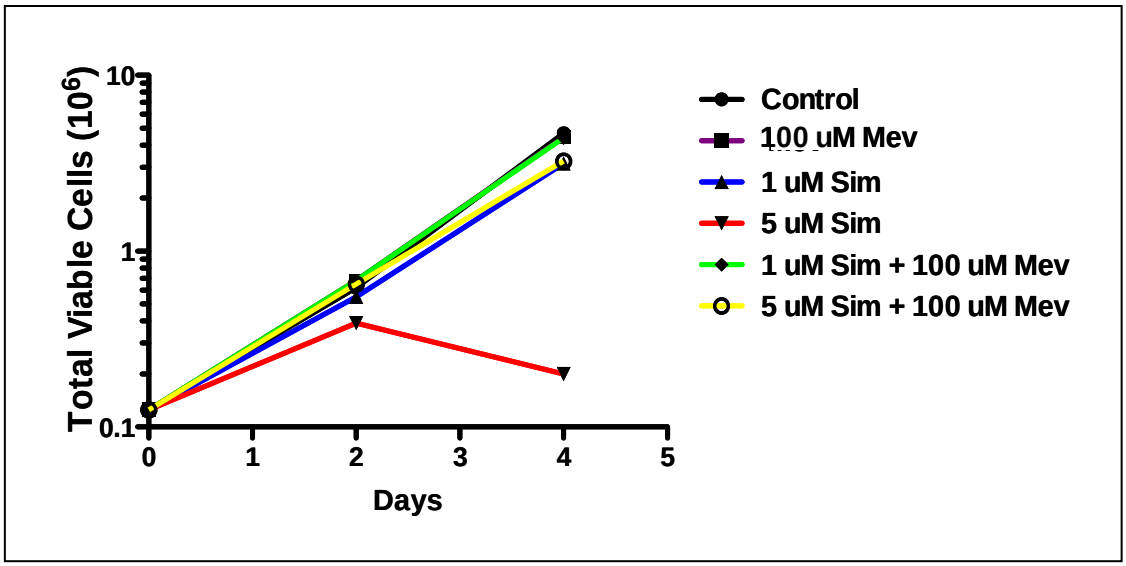


Figure 17: Mevalonate restores cytotoxic effects induced by simvastatin in HEL cells. HEL cells were treated with 0 (0.1% DMSO and 0.1% methanol), 100 µM mevalonate (Mev), and 1 or 5 µM simvastatin (sim) alone, or in combination with 100 µM Mev were counted every two days using trypan blue exclusion. Mev prevented the cytotoxic effect of sim treatment. Data represents a single experiment that was replicated three times.

To investigate the possibility that farnesylpyrophosphate (FPP) could restore the minor inhibition on farnesylation we detected with 5 µM simvastatin and 5 µM lovastatin, we treated HEL cells with 5 µM simvastatin and 10 µM FPP for 48 hours and assessed HDJ-2. Western blot analyses showed that FPP did restore HDJ-2 farnesylation as demonstrated by the single HDJ-2 band (Figure 18B), suggesting FPP is functional and warrants use in studies that aim to reverse the cytotoxic effects of simvastatin.

Farnesylpyrophosphate does not readily restore viable cell numbers after statin treatment in JAK2-V617F-driven cell lines

Although we detected only a minor inhibition of farnesylation after simvastatin treatment, we sought to rule out the possibility that statins cytotoxic actions were due to targeting farnesylation to any extent. To restore the defect in farnesylation we added back farnesylpyrophosphate (FPP) to HEL cells treated with 5 µM simvastatin and found there to be a partial restorative effect on total viable cell numbers (Figure 19A) and viability over time (Figure 19B), suggesting that FPP does not readily prevent the cytotoxic effects on viable cell numbers and viability induced by 5 µM simvastatin in HEL cells.

Effects on geranylgeranylation after statin treatment in JAK2-V617F-driven cells

To investigate components further downstream of FPP in the mevalonate pathway, we assessed the state of geranylgeranylation after statin treatment in HEL cells. We used Rap1A protein as a tool to gauge geranylgeranylation, for it is exclusively geranylgeranylated. Using an antibody that recognizes the Rap1A carboxy terminus, which gets cleaved after geranylgeranylation, and thus this antibody only recognizes unprenylated Rap1A protein. We demonstrated that sim, lov, and ator induced the accumulation of unprenylated Rap1A. This demonstrated clear inhibition of geranylgeranylation. As a control to induce unprenylated Rap1A protein, we utilized the geranylgernanyl transferase inhibitor GGTI-2417 (Figure 20A). Furthermore, we reversed the inhibition on geranylgeranylation with geranylgeranylpyrophospahte (GGPP) as shown by

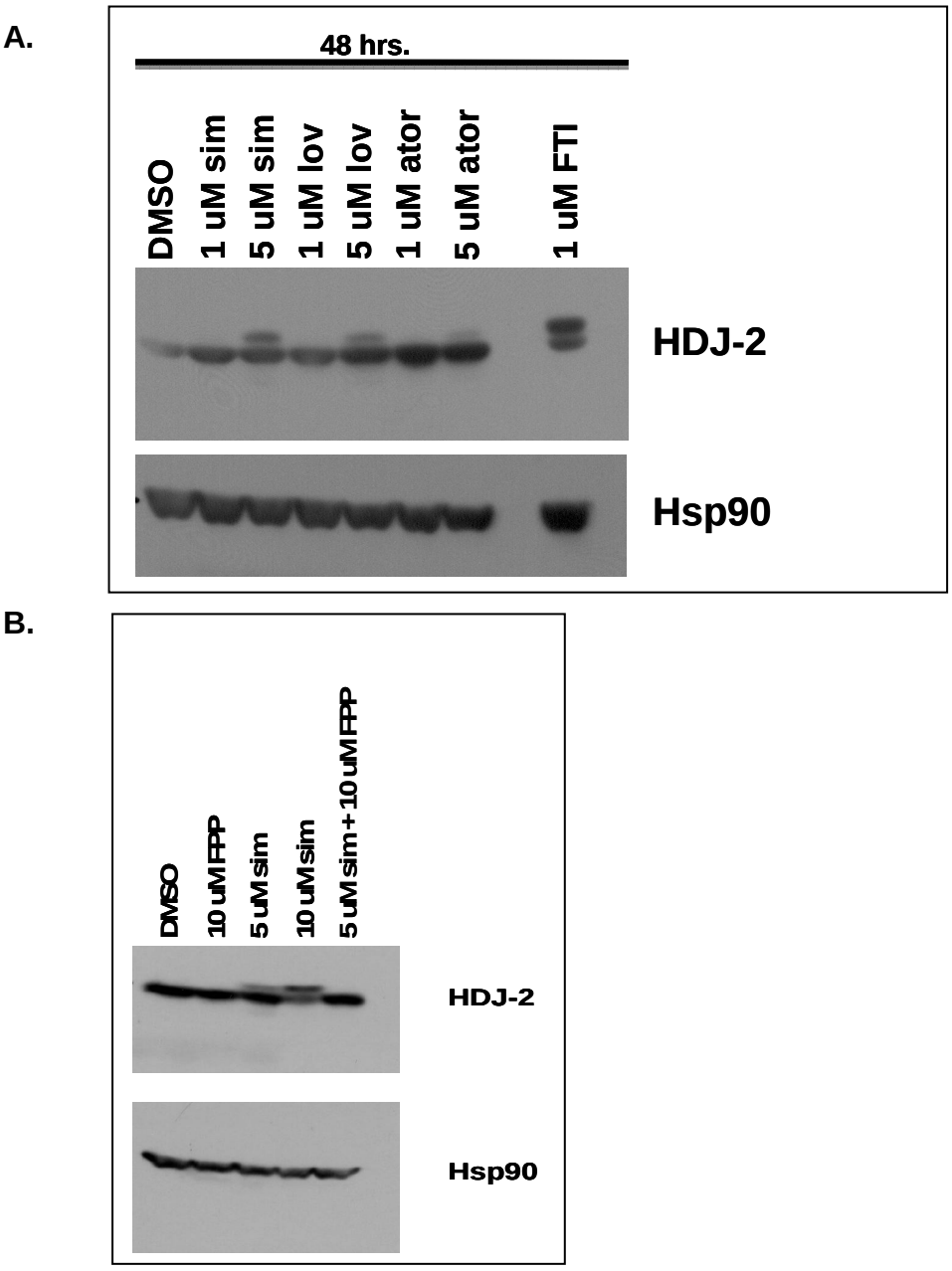


Figure 18: Statins induce a minor inhibition on farnesylation in HEL cells. (A) HEL cells were treated with 0 (DMSO), 1 or 5 µM simvastatin (sim), lovastatin (lov), or atorvastatin (ator) for 48 hours. Western blot analysis of HDJ-2, a protein that is exclusively farnesylated, showed that statins induced minor inhibition of farnesylation as shown by the appearance of the double band (unprenylated and prenylated HDJ-2). HEL cells were also treated with 1 µM FTI-2153 (farnesyl transferase inhibitor) as a positive control for inhibition of farnesylation. (B) Western blot analysis of HEL treated with farnesylpyrophosphate (FPP) and 5 µM sim for 48 hours demonstrated restoration in HDJ-2 farnesylation (single band indicates active farnesylation), suggesting FPP add back does restore minor farnesylation defects induced by sim.

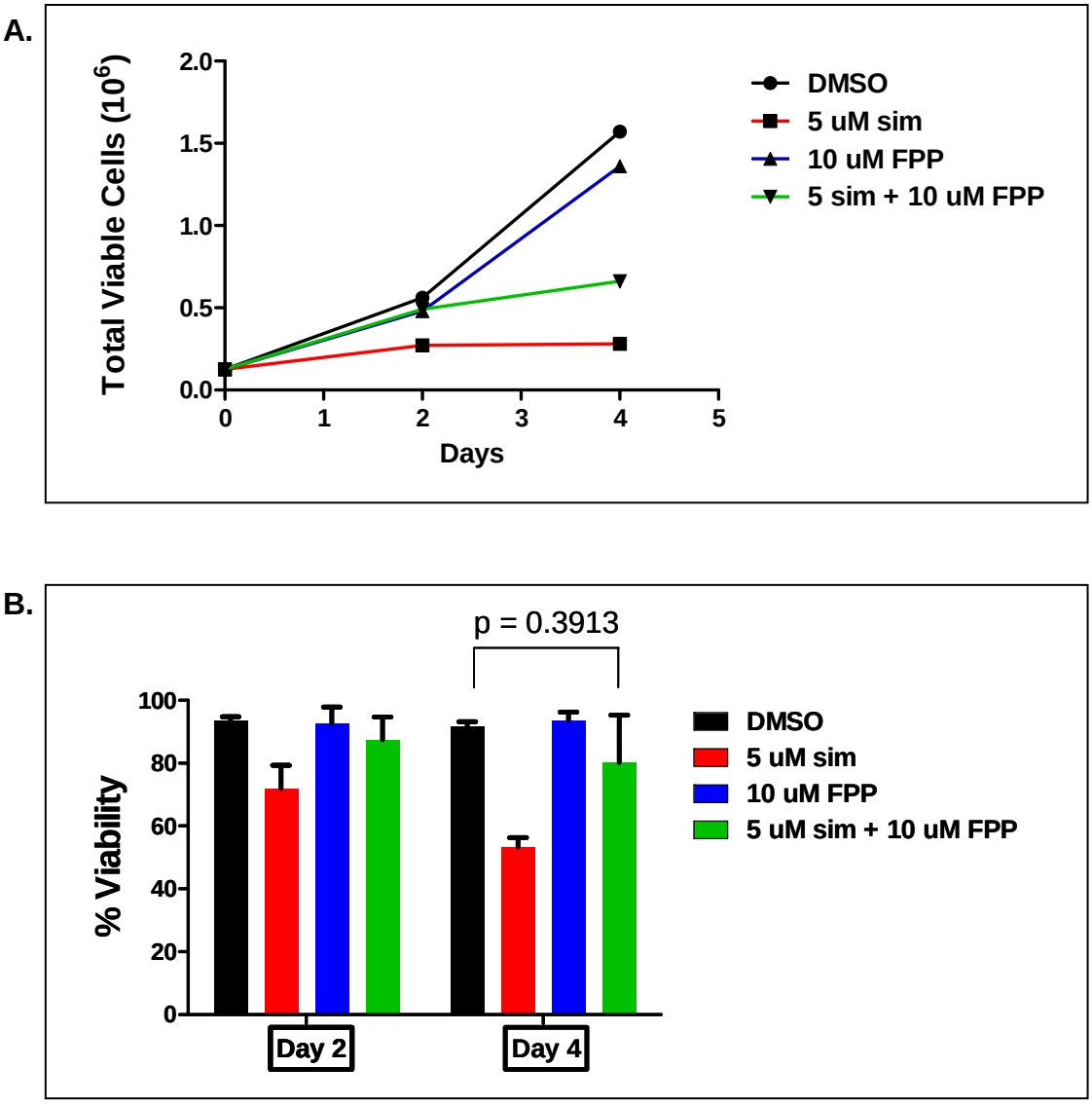


Figure 19: Farnesylpyrophosphate (FPP) partially restores cytotoxic effects induced by simvastatin in HEL cells. (A) HEL cells were treated with 0 (DMSO), 5 µM simvastatin (sim), and 10 µM FPP alone or in combination with 5 µM sim and counted with trypan blue. FPP addition with 5 µM sim resulted in a slight restoration in total viable HEL cells over time. (B) The viability of growth curve samples was analyzed (# of live cells/# of total cells) and showed that FPP in combination with 5 µM sim partially restored cell viability compared to 5 µM sim alone. Error bars represent standard deviation and p value calculated using T-test (GraphPad Prism, Inc.). Experimental data represents a single experiment which was replicated three times, with replicates supporting presented data.

western blot analysis of Rap1A in HEL cells treated with 5 µM simvastatin and 10 µM GGPP for 48 hours (Figure 20B).

GGPP partially restores total viable HEL cells treated with simvastatin after 48 hours of treatment, with loss of any restorative effect at 96 hours

To determine if the inhibition of geranylgeranylation was responsible for statin's cytotoxic effects, we performed experiments adding back geranylgeranyl pyrophosphate (GGPP) to statin treated HEL cells and assessed viable cell numbers over time. Interestingly, GGPP could not restore cell growth (Figure 21A). However, the viability of HEL cells treated with simvastatin was restored after 48 hours of treatment with GGPP add back, but at the 96 hour time point and beyond, no restorative effect was observed (Figure 21B).

Assessing the role of combined prenylation defects in statin action in targeting JAK2-V617F-driven cells

To address the effects on combined prenylation, both farnesylation and geranylgeranylation, after statin action in JAK2-V617F-driven cells, we performed add back experiments restoring GGPP and FPP simultaneously and used trypan blue exclusion analysis to determine viable cell numbers. The combination of 10 µM GGPP and 10 µM FPP co-cultured with 5 µM simvastatin treated HEL cells did not improve total viable cell numbers compared to the 5 µM simvastatin and 10 µM FPP treated sample, or the 5 µM simvastatin and 10 µM GGPP sample (Figure 22).

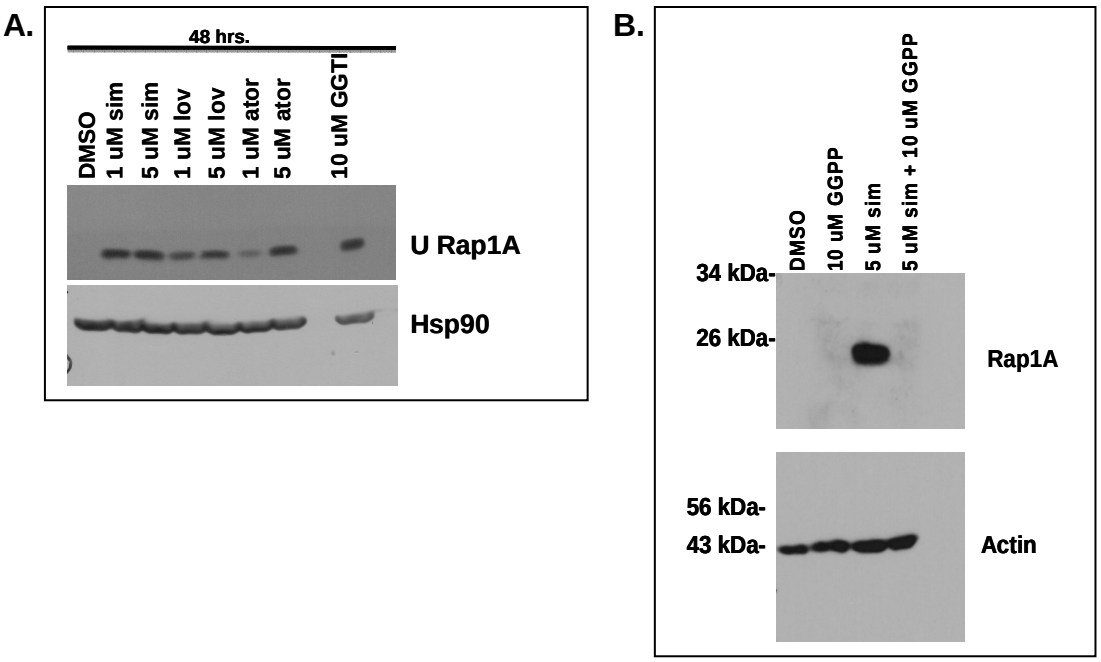


Figure 20: Statins inhibit geranylgeranylation in JAk2-V617F-dependent cells. (A) HEL cells were treated with 0 (DMSO), 1 or 5 µM simvastatin (sim), lovastatin (lov), or atorvastatin (ator) for 48 hours and analyzed by western blot to assess inhibition of geranylgeranylation by probing with an antibody that recognizes the carboxy terminus of Rap1A, which gets cleaved after geranylgeranylation. We treated HEL cells with 10 µM GGTI-2417 (geranylgeranyl transferase inhibitor) for 48 hours, as a positive control for geranylgeranylation inhibition (presence of band indicates inhibition). Sim, lov, and ator inhibited geranylgeranylation. Hsp90 was detected as a loading control. (B) To test if the inhibition on geranylgeranylation could be reversed with geranylgeranylpyrophosphate (GGPP), we ran western blot analysis of HEL cells treated with 0 (DMSO), 5 µM sim alone, and 10 µM GGPP alone or in combination with 5 µM sim and by probing for Rap1A. GGPP restored the inhibition of geranylgeranylation induced by simvastatin. Actin was detected as a loading control.

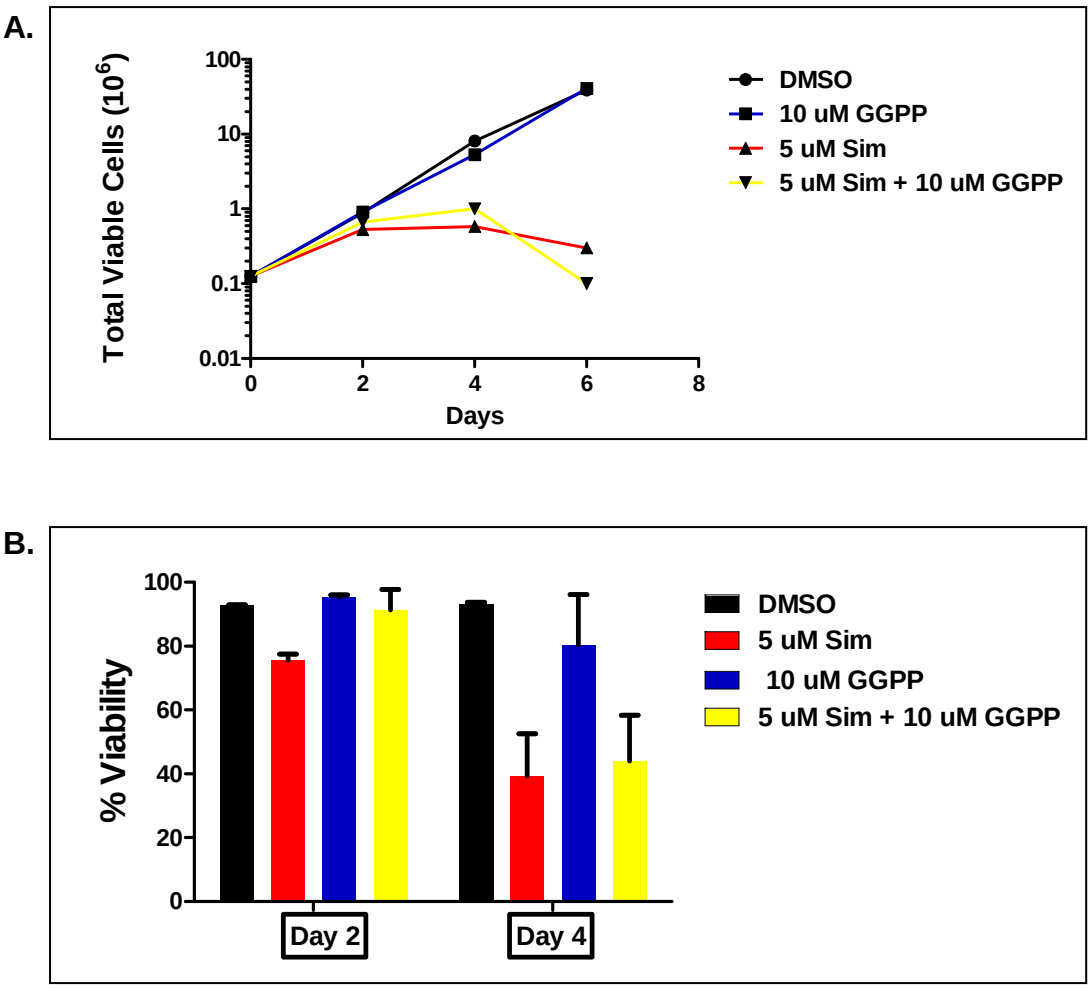


Figure 21: GGPP addition can not restore HEL cell growth and viability following simvastatin treatment. (A) HEL cells were treated with 0 (DMSO), 5 µM simvastatin (sim), 10 µM GGPP, alone or in combination with 5 µM sim and counted with trypan blue. GGPP does not restore viable HEL cell numbers over time. (B) Analysis of the viability of HEL cells treated with sim, GGPP alone, or in combination demonstrated that viability was restored at short-time points (Day 2), but restorative effect was lost at later time points (Day 4). Error bars represent standard deviation of duplicate samples. Data represents experiments that were run a minimum of three times.

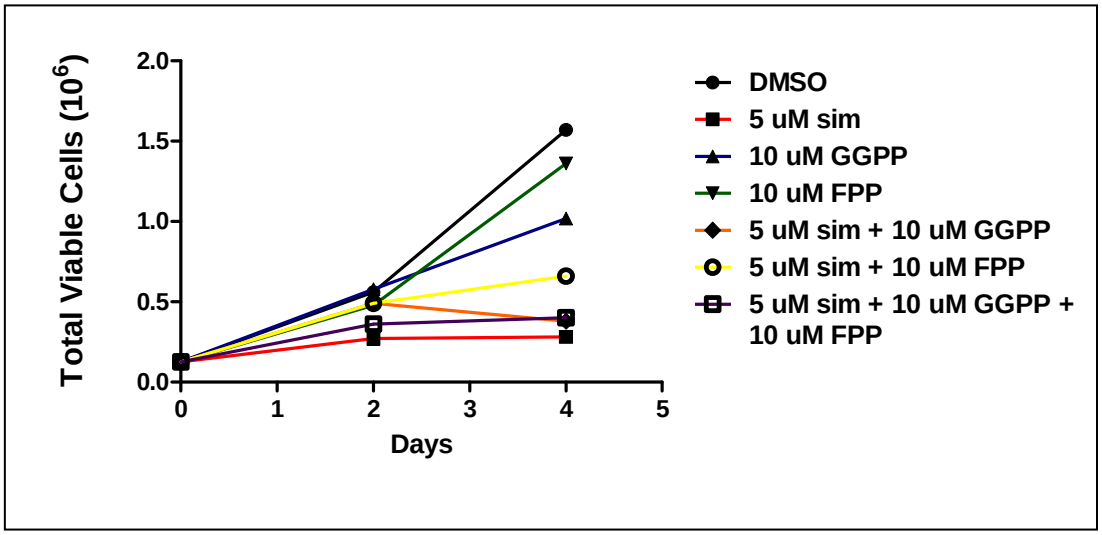


Figure 22: Restoration of prenylation in simvastatin treated HEL cells does not restore viable cell numbers. HEL cells were treated with 0 (DMSO), 5 µM simvastatin (sim), 10 µM GGPP, and 10 µM FPP alone and in combination as indicated, and counted with trypan blue. Add back of both prenylation end products, FPP and GGPP, did not restore viable cell numbers when compared to the 5 µM sim and DMSO controls. This suggests that overall prenylation may not be solely responsible for the cytotoxic effects induced by sim. Data represents a single experiment that was run in triplicate.

The effect of simvastatin on EpoR glycosylation

Hamadmad et al. previously reported lov treatment at short-time points could downregulate surface expression of EpoR in ASE2, an erythroleukemia cell line [203]. Hamadmad et al. demonstrated that lov treatment inhibited geranylgeranylation and dolichol, a downstream intermediate off of the geranylgeranyl branch of the mevalonate pathway involved in N-linked glycosylation with proteins like EpoR (refer to Figure 6). Furthermore, due to EpoR potentially playing an important role in PV and because JAK2-V617F requires a homodimeric cytokine receptor to function, we questioned if simvastatin and its ability to inhibit geranylgeranylation, affected EpoR glycosylation. After treating HEL cells with 5 µM simvastatin for 48 hours, we

found a difference in the expression pattern of EpoR by western blot analysis. HEL cells treated with DMSO control displayed a two-band protein banding pattern with a ~66 kDa (mature, fully gycosylated form) and ~64 kDa (maturing form) band (Figure 23; lane 1), while the 5 µM sim treated sample displayed a prominent ~64 kDa (maturing form of EpoR) with possible breakdown products of EpoR between 34 and 43 kDa (Figure 23; lane 3). We have confirmed that the ~66kDa form is fully gylcosylated because of its resistance to deglycosylation by EndoH (data not shown), a characteristic of full glycosylation. The ~66kDa fully gycosylated form is utilized as a surrogate for plasma membrane localized EpoR and thus sim treatment appears to inhibit plasma membrane localization of EpoR. We were also able to confirm that restoring geranylgeranylation with 10 µM GGPP after 5 µM sim treatment in HEL cells did restore the expression of the ~66 kDa EpoR band (mature form) (Figure 23; lane 4).

Discussion

Statin drugs have recently developed an appreciated potential in the treatment of some cancers including melanoma [239], glioma [240], neuroblastoma [241], prostate cancer [170, 242-243], and leukemia [234, 244]. We were the first to report on the anti-neoplastic effects of statins in JAK2-V617F-driven myeloprolifertaive cell lines and primary MPN samples [238]. The complete mechanism of action behind statin activity in the hypercholesteremia setting is still not fully elucidated. Statins have been shown to have a multitude of effects

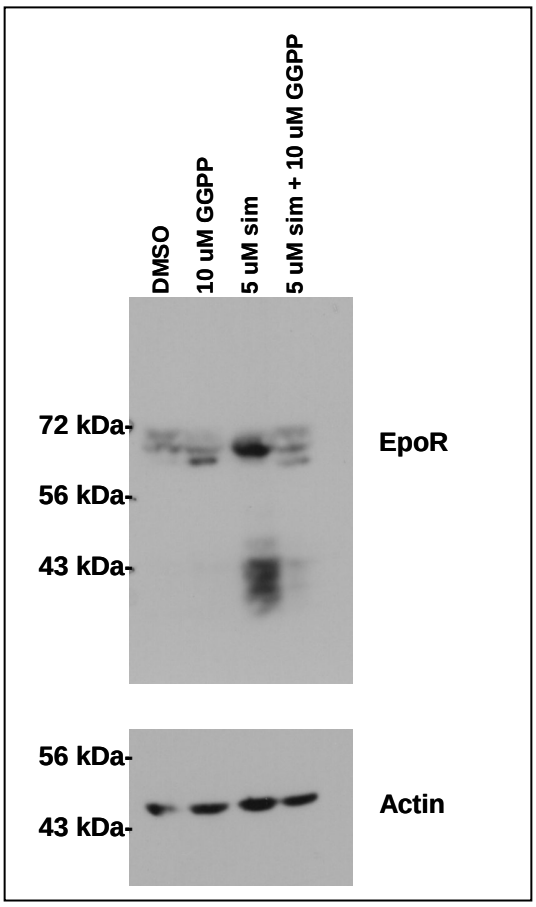


Figure 23: Simvastatin alters expression of EpoR gycosylation forms in HEL cells. HEL cells were treated with 0 (DMSO), 10 µM GGPP, and 5 µM simvastatin (sim) alone, or in combination with 10 µM GGPP for 48 hours and were analyzed by western blot to assess EpoR expression. Sim treatment promoted an accumulation of the 64 kDa EpoR form (partially glycosylated, still maturing form) whereas, the DMSO control displayed the fully glycosylated EpoR (~66 kDa) and the maturing EpoR form (64 kDa), suggesting sim blocked EpoR from becoming fully glycosylated. Sim also induced potential breakdown products of EpoR with detection of multiple bands ranging in molecular weights from ~20-56 kDa. To test the effects of reduced GGPP and subsequent loss of dolichol, GGPP was co-cultured with 5 µM sim treated HEL cells for 48 hours and analyzed by western blot to investigate EpoR glycosylation isoforms. GGPP restored the fully glycosylated EpoR isoform (~66 kDa).

that contribute to lowering cholesterol in cardiovascular risk patients [134, 153, 224, 226-227]. Variability of statin action can be influenced by cell type, disease type, type of statin, dosage, and duration of treatment [245]. In this work, we further investigated the statin mechanism of action in the setting of JAK2-V617F-driven transformation. Indeed, 1 and 5 µM doses of all statins tested (sim, lov, and ator) that induced cytotoxic effect in JAK2-V617F-driven cell lines reduced cholesterol to variable capacities depending on the statin and dose. Since our previous work showed a dependence of JAK2-V617F signaling on lipid rafts, we initially chose to treat the JAK2-V617F-dependent MPN model cell lines with statins in an attempt to alter lipid rafts, which are rich in and are dependent on cholesterol. Statins, like lipid raft disrupting agents we hoped would alter plasma membrane cholesterol and hence disrupt lipid rafts, the site where JAK2-V617F protein complexes may be located. Although our data did not provide evidence that 5 µM ator disrupted lipid rafts, we did find decreased JAK2 protein in the raft fractions, suggesting that JAK2 protein and lipid raft co-localization was disrupted. Considering the percentage of cholesterol reduction after atorvastatin treatment (46% reduction after 96 hours of treatment), in comparison to lipid raft disrupting agents, which are thought to drastically alter cholesterol, statins may not disrupt lipid rafts enough to the level of detection in the lipid raft isolation assay. Nonetheless, we demonstrate evidence for an alteration in JAK2 protein localization with lipid rafts, but no direct evidence for lipid raft disruption by atorvastatin treatment. Interestingly, cholesterol add back allowed for only a partial restoration in total viable SET-2 cells over time, suggesting that the

cholesterol decrease may not be solely responsible for the cytotoxic effects induced by statins. It is important to note that few reports that attempt to analyze statin affects by adding back products of the mevalonate pathway ever demonstrate that cholesterol add back is successful. In fact, personal communication with other researchers has confirmed that cholesterol addition can be toxic to cells, as we have seen when elevated cholesterol addition is used [246]. These results lead us to examining potential effects on end products other than cholesterol in the mevalonate pathway.

We first took a step back and added an early intermediate of the mevalonate pathway, mevalonate itself. Mevalonate is produced by the activity of HMG-CoA reductase and thus its levels should be decreased following treatment with statins, which are inhibitors of HMG-CoA reductase (Figure 17). Indeed, mevalonate completely rescued the growth inhibitory effects of statin treatment, demonstrating that, the HMG-CoA reductase inhibitory activity of statins is responsible for the detrimental effects observed on JAK2-V617F-driven cell growth (Figure 17).

Since statin treatment clearly inhibited geranylgeranylation (Figure 20) we also performed GGPP add back experiments. GGPP add back had a slight restorative effect on the viability of HEL cells in the short-term but not the long-term (Figure 21). Indeed, simvastatin inhibited geranylgeranylation in these JAK2-V617F-driven cells, but GGPP add back could not restore growth, suggesting that inhibition of geranylgeranylation is not solely responsible for the cytotoxic effect induced by simvastatin. Perhaps the initial cell death induced by

simvastatin is due to geranylgeranylation inhibition but additional effects of inhibiting the mevalonate pathway cannot be overcome by GGPP, and this leads to the inability of GGPP to restore long-term growth and viability. This is not inconsistent with other published work, which suggests GGPP add back can rescue the effect of statin treatment. Such data are generally always presented as short-term experiments and never address long-term effects such as the ability to restore cell growth. Additionally, simvastatin elicits a minor inhibition of farnesylation (Figure 18). Combination add back of the isoprenoid intermediates FPP and GGPP, was also unable to fully restore cell growth and viability after simvastatin treatment, suggesting statin's cytotoxic effect is not based on inhibiting prenylation alone. However, the inhibition of geranylgeranylation was shown to be responsible for the redistribution of the glycosylated forms of EpoR, shifting the EpoR protein population to the immature glycosylated isoform. Adding back GGPP restored fully processed/glycosylated (plasma membrane localized) EpoR (Figure 23). Since JAK2-V617F requires a cytokine receptor for signaling, this could be a mechanism by which statins may affect JAK2-V617F signaling, that is, statins may regulate the processing and subsequent plasma membrane localization of a requisite cytokine receptor. However, in the cell lines we have utilized, the specific requirement for EpoR remains unknown, but it is possible the glycosylation of other receptors may be similarly affected by statin treatment. Because mevalonate completely protected cells from the effects of statin treatment (Figure 17), we conclude that the effects of statins are indeed due to its inhibition of the mevalonate pathway. However, since isoprenoid

intermediate add back did not protect cells from the effects of statin treatment, it is likely that inhibition of other pathways or a combination of pathways that are dependent on products of the mevalonate pathway are responsible for the effect of statins on JAK2-V617F-driven cell growth.

In summary, we have shown statins affect multiple components of the mevalonate pathway, including inhibition of cholesterol biosynthesis, geranylgeranylation, and a minor inhibition of farnesylation, in MPN model cells that are dependent on JAK2-V617F. Statins presumably inhibit the various end products of the mevalonate pathway and induce a variety of cellular changes. These changes may no longer be reversible or occur at a rate that is not compatible with add back of the end products, as we were unable to identify a single end product whose inhibition could be determined to elicit the effects of statin treatment. While the exact mechanism by which statins induce its cytotoxic effects on JAK2-V617F-positive MPN model cells remains to be elucidated, it is clear that these cells are sensitive to statins by undergoing apoptosis which results in decreased cell growth. This suggests statin treatment may be an option to incorporate into future therapies for MPNs, including possible combination with JAK2 inhibitors.

Materials and Methods

Growth curves

HEL or SET-2 cells were plated at a concentration of 0.1 – 0.25 X 10^6 cells/mL and treated with 0 (DMSO), and 1 or 5 µM simvastatin (Sigma-Aldrich

#S6196). 1X synthechol (Sigma-Aldrich # S5442), 100 µM mevalonate (Sigma-Aldrich #79849), 10 µM FPP (Sigma-Aldrich # 6892), and 10 µM GGPP (Sigma-Aldrich #G6025) were added alone, or in combination (DMSO content maintained at 0.1% across all samples) and cell counts and viability were assessed every two days using trypan blue. Cell cultures were passed every two days with replenishment of additive lipid compounds, ensuring the stability of these lipids is not a concern in the experiments.

Cholesterol assay

Cholesterol was measured using Amplex Red Reagent as described in Chapter 2 [238]. Briefly, 2 – 3 X 10^6 cells were treated with 0 (DMSO), 1 or 5 µM simvastatin (Sigma-Aldrich #S6196), lovastatin (Sigma-Aldrich #M2147), or atorvastatin (Sigma-Aldrich #PZ0001) for 96 hours. Cells were washed with 1X RT PBS three times and ficolled with lymphocyte separation media (Corning #25-072-CV) to remove dead cells. Amplex Red Cholesterol Measurement kit (Invitrogen #A12216) was used to measure cholesterol as manufacture recommends. Fluorescence was measured using Synergy HT fluoremeter BioTek Inc. Winooski, VT, USA) using 560/590 excitation/emission settings.

Western blot

2 – 5 X 10^6 cells were washed with 4ºC 1X PBS and lysed in 4ºC lysis buffer (25 mMTris, pH=7.4, 150 mM NaCl, 25 mM NaF, 1% Triton-X-100, and protease inhibitors). Cell lysate was centrifuged at 14.5 X 10^5 X g for 5 minutes at

4ºC. Pierce BCA reagent (Thermo Scientific) was used to determine protein concentration. Protein lystaes were run on SDS-PAGE (8 - 12.5% gels). Primary antibodies included: total JAK2 (Cell Signaling Technology #3230), Rap1A (Santa Cruz #sc-1482), HDJ-2 (Neomarker #AM00209PU-N), Hsp90 (Santa Cruz #sc-7947), actin (Sigma #AC-74), EpoR (Amgen A82), Flotillin-1 (Cell Signaling Technologies #3253), and CTB-HRP (Sigma-Aldrich #C3741). Secondary antibodies were from Thermo Scientific. West Pico Chemilluminescence, ECL Plus, or West Femto Chemilluminescence were used to develop western blots. DRM dot blot and western blot analyses performed in same fashion as described in Chapter 2 [238].

Chapter 4

Statins cooperate with JAK inhibitors to enhance the killing effect on JAK2-V617F-driven cells

Introduction

The classical MPNs including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), are hematopoietic stem cell disorders characterized by expansion of the myeloid lineage in one form or another [186]. Dysregulated JAK/STAT signaling is common to the classical MPNs [65, 95], with activating mutations found in patients including mutations in cytokine receptors (i.e. MPL-W515) [78-79], JAK2 (i.e. JAK2-V617F [186], exon 12 mutations [74]) as well as inactivating mutations in negative regulators of JAK/STAT signaling (e.g. SOCS1, LNK, etc.) [64].

The JAK inhibitor, ruxolitinib (Incyte, INCB018424) was recently approved for the treatment of MF in November of 2011. Ruxolitinib induced a marked reduction in splenomegaly and reduced in constitutional symptoms including fever, fatigue, cachexia, night sweats, anemia, pruitus, and bone pain [247]. However, JAK inhibitors have not been shown to induce partial or complete remissions, nor have they been shown to reduce allele burden in MF patients [94, 205], suggesting there is much room for improvement in the treatment of MPN patients. It is well established that JAK/STAT signaling is defective in MPNs

[65], and it is plausible that targeting multiple sites in a key, dysregulated pathway may be necessary to effectively block oncogenic signaling.

We have demonstrated that cholesterol-lowering statins reduce total viable cell numbers and induced apoptosis in JAK2-V617F-driven cell lines and inhibit colony formation of primary MPN cells (Chapter 2). In our current study, we hypothesized that statins will enhance the cytotoxic effect of JAK2 inhibition on JAK2-V617F-dependent cells. We demonstrate an enhancement of cytotoxic effects when combining statin therapy with JAK inhibitor therapy in JAK2-V617F-driven cells. We show statin and JAK inhibitor combination treatment enhances the reduction in total viable cell numbers and viability over time compared to single agent treatment alone. Additionally, we show that combination treatment amplifies the number of cells undergoing apoptosis compared to single agent treatment. Furthermore, we found that combination treatment led to a G1 arrest in the cell cycle. Our work is the first to provide evidence that statins drugs cooperate with JAK inhibitors in JAK2-V617F-driven cells and proposes statins may enhance JAK inhibitor therapy in patients.

Results

To determine if there is a potential therapeutic advantage to combining statin treatment with JAK inhibitors, we treated a panel of JAK2-V617F-driven MPN model cells. These cell lines include HEL (Human Erythroleukemia) cells, homozygous for the JAK2-V617F mutation, SET-2 cells, heterozygous for the

JAK2-V617F mutation, and Uke1 cells, homozygous for the JAK2-V617F mutation [196].

Simvastatin cooperates with JAK Inhibitor I to enhance the reduction in viable HEL cells

HEL cells treated with simvastatin or JAK Inhibitor I (JI), a pan JAK inhibitor, were effective at reducing total viable cell numbers as single agents, but in combination led to an enhanced reduction in total viable cell number over time (Figure 24A). Calculated fold reductions comparing treated samples to DMSO control (DMSO total viable cell number/drug-treated sample total viable cell number = fold reduction) were also graphed (Figure 24B). HEL cells treated with 1 µM sim alone showed no reduction in total viable cells compared to DMSO control at day 4, whereas 1 µM JI led to a 11.7 fold reduction. Combination of 1 µM sim and 1 µM JI resulted in 17.5 fold reduction in total viable cells. Likewise, 5 µM sim alone resulted in a 1.9 fold reduction at day 4, while combining 5 µM sim with 1 µM JI led to a 50.9 fold reduction in total viable HEL cells (Figure 24B). Interestingly, the 1 µM JI sample after eight days of treatment demonstrated signs of resistance with increases in the total viable cell numbers (Figure 24A). However, JI combined with only 1 µM simvastatin eliminated resistance from occurring (Figure 24A).

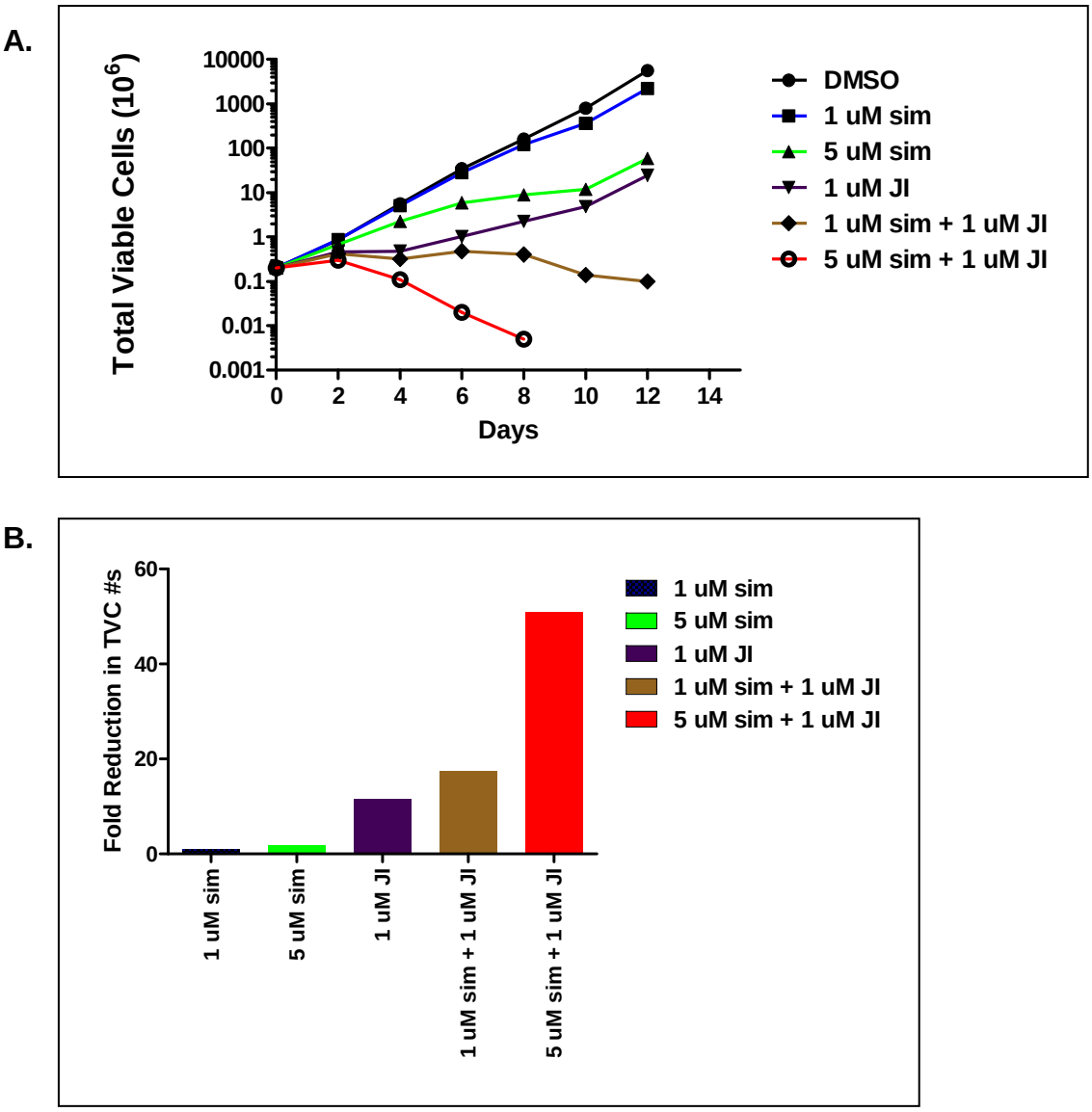


Figure 24: Simvastatin cooperates with JAK Inhibitor I in HEL cells to enhance the reduction in total viable cell numbers. (A) HEL cells were treated with DMSO (0.1% dimethyl sulfoxide), low dose simvastatin (sim), and JAK Inhibitor I (JI) alone, and in combination and counted every two days using trypan blue exclusion. Low dose sim and JI alone reduce total viable cell numbers, but when combined led to an enhanced reduction in cell numbers over time. (B) Graph represents total viable cell fold reduction of drug treated samples compared to DMSO control at day 4 (Equation: (DMSO total viable cell number/ sample total viable cell number = fold reduction). Experimental data represents a single experiment which was replicated in SET-2 cells (Figure 25).

Simvastatin cooperates with JAK Inhibitor I to enhance the reduction in viable SET-2 cells

Similarly, in a second JAK2-V617F-dependent cell line, SET-2 cells, displayed a 2 fold reduction in total viable cell numbers in response to 5 µM simvastatin treatment after 4 days of treatment (Figure 25A). 5 µM simvastatin alone, led to a 2 fold reduction, while, 1 µM JI led to a 11.9 fold reduction at day 4. When combined, these two drugs induced a 23.7 fold reduction in total viable cells after 4 days and this reduction continued to increase over additional days of culture. Impressively, 5 µM sim with 1 µM JI led to 83 fold reduction in total viable cells compared to DMSO control (Figure 25B). These data demonstrate that the cooperative ability of simvastatin and JAK Inhibitor I are not JAK2-V617F-positive cell line dependent, as both HEL (Figure 24) and SET-2 (Figure 25) cells responded similarly.

Simvastatin cooperates with INCB018424 to enhance the reduction in viable HEL cells

To test the potential of a second JAK inhibitor to cooperate with statins, we tested the effects of the JAK1/2 inhibitor, INCB018424 (424, ruxolitinib) combined with statin treatment on total viable cell numbers and % viability as demonstrated by trypan blue exclusion. Concordant with the HEL data, 1 µM simvastatin combined with 0.25 µM 424 led to a similar decrease in total viable HEL cell numbers (Figure 26A) (5.6 fold reduction) as 0.5 µM 424

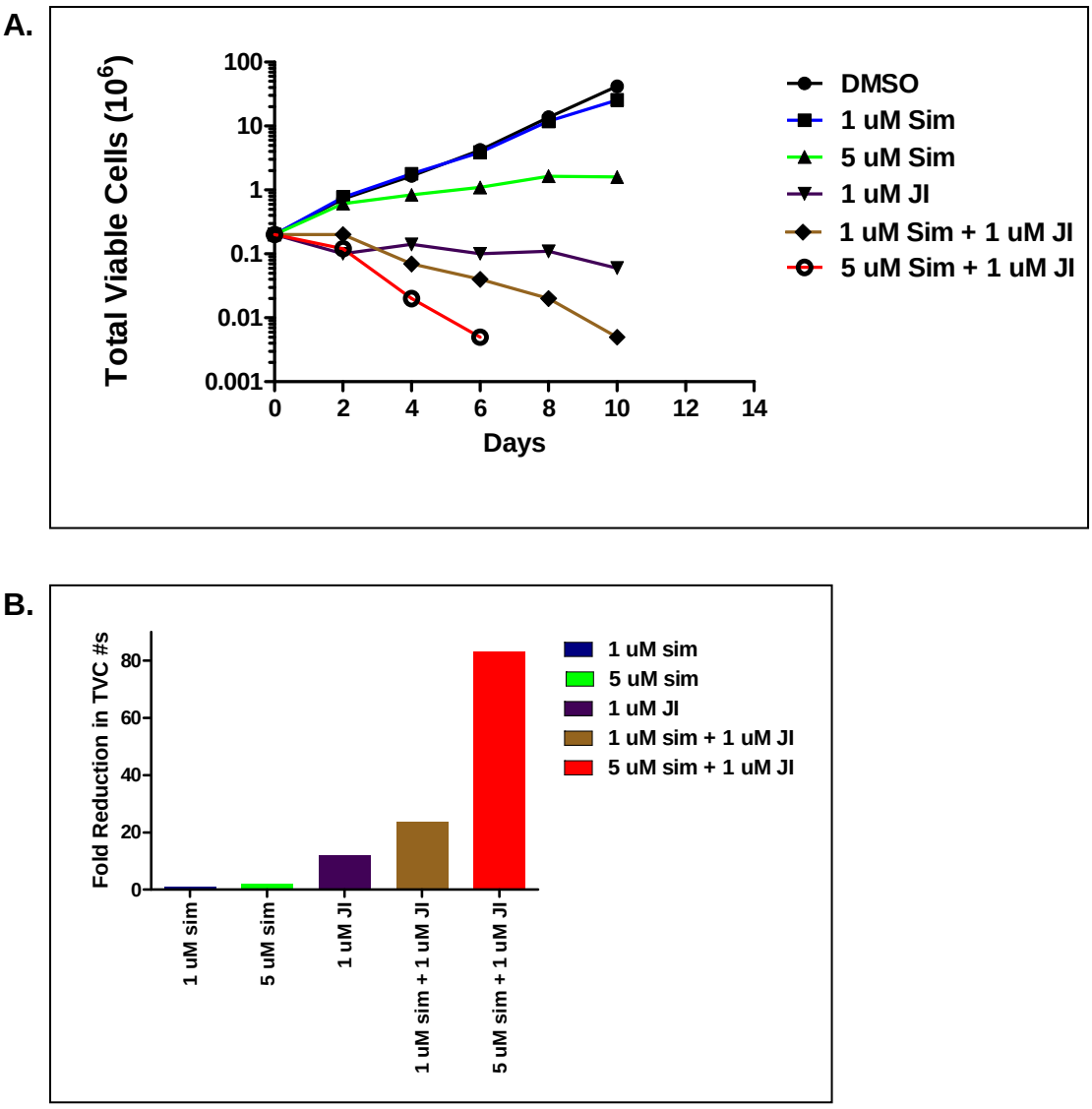


Figure 25: Simvastatin cooperates with JAK Inhibitor I in SET-2 cells to enhance the reduction in total viable cell numbers. (A) SET-2 cells were treated with DMSO, low dose simvastatin (sim), and JAK Inhibitor I (JI) alone, and in combination and counted every two days using trypan blue exclusion. Low dose sim and JI alone reduce total viable cell numbers, but when combined lead to an enhanced reduction in cell numbers over time. (B) Graph represents total viable cell fold reduction comparing drug treated samples to DMSO control on day 4. Experimental data represents a single experiment which was replicated in HEL cells (Figure 24).

treatment alone (5.9 fold reduction) (Figure 26B). Enhanced fold reductions were seen when combining 1 µM sim (1.6 fold reduction alone) with 0.5 µM 424 (5.9 fold reduction alone), resulting in a 10.9 fold reduction in viable cells. Enhanced growth inhibition of the culture was especially apparent in later time points of the experiment (e.g. day 10).

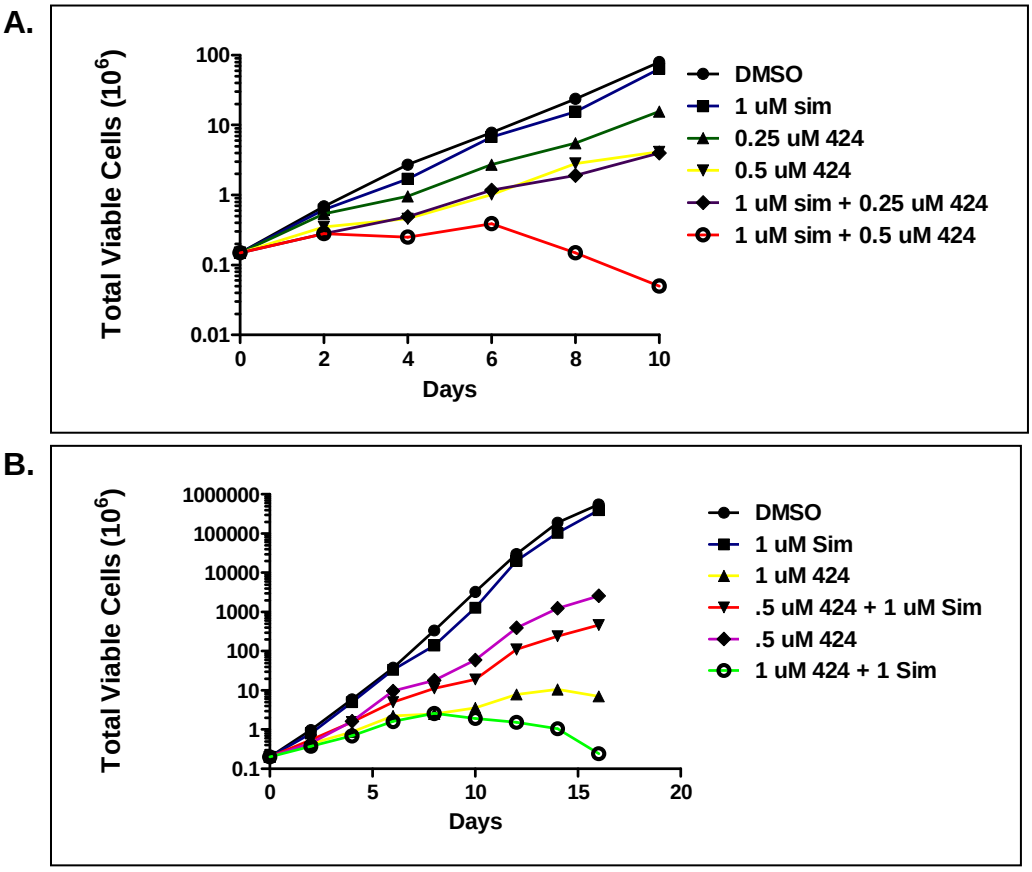


Figure 26: Simvastatin cooperates with INCB018424 in HEL cells to enhance the reduction in total viable cell numbers. (A) HEL cells were treated with low dose simvastatin (sim) or INCB018424 (424) alone, or in combination and were counted every two days using trypan blue. Combination treatment of sim and 424 lead to an exaggerated reduction in total viable cell numbers over time, in a dose-dependent manner. (B) Replicate growth curve to 26A.

C.

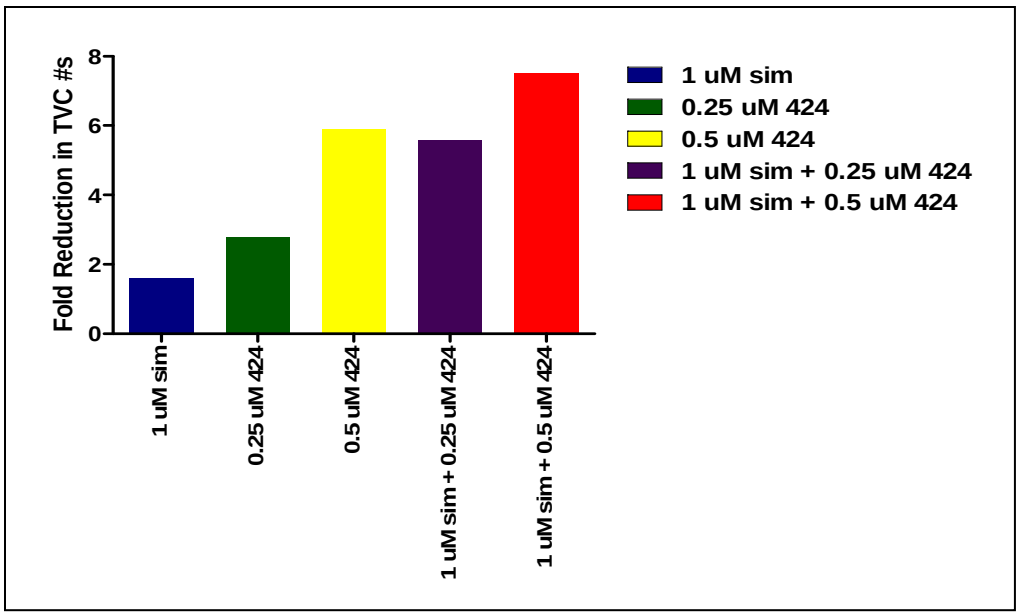


Figure 26 (continued): Simvastatin cooperates with INCB018424 in HEL cells to enhance the reduction in total viable cell numbers. (C)Graph represents total viable cell fold reduction of drug treated samples compared to DMSO control on day 4. Experimental data represents a single experiment which was replicated in SET-2 cells (Figure 27), and multiple statins (Figure 28 & 29), with replicates supporting presented data.

Simvastatin cooperates with INCB018424 to enhance the reduction viable SET-2 cells

SET-2 cells displayed similar enhanced cytotoxic effects with the combination of simvastatin and INCB018424 treatment as shown by the reduction in total viable cell numbers (Figure 27A). SET-2 cells treated with 1 µM sim resulted in a subtle 1.4 fold reduction in viable cells at day 4, while 0.25 µM 424 led to a 3.1 fold reduction. Combining 1 µM sim with 0.25 µM 424 gave rise to an enhanced 5 fold reduction at day 4. Again, combining 1 µM sim with 0.5 µM 424 (4.5 fold reduction with 0.5 µM 424 alone) resulted in an 8.8 fold reduction in viable cells at day 4 (Figure 27B).

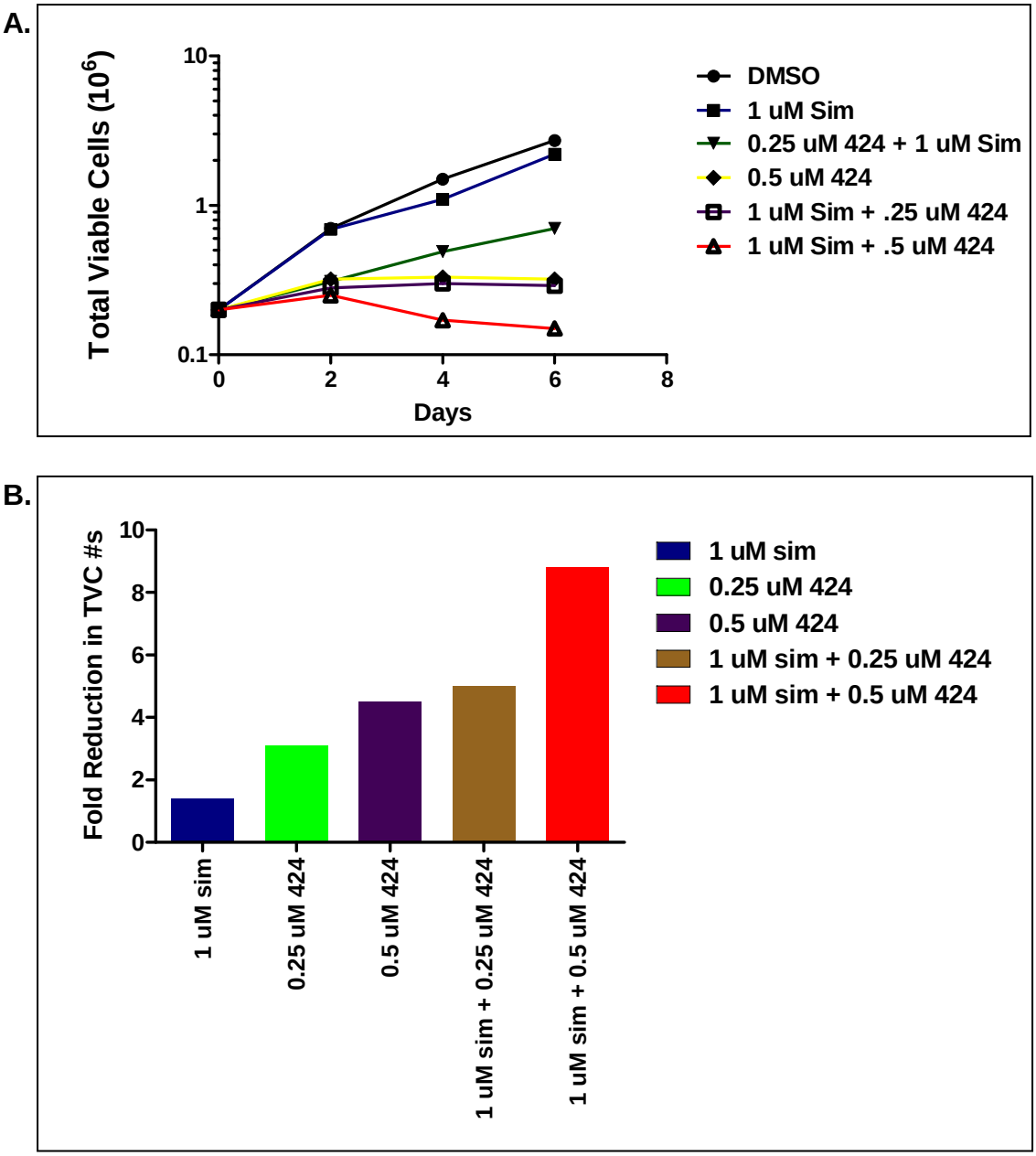


Figure 27: Simvastatin cooperates with INCB018424 in SET-2 cells to enhance the reduction in total viable cell numbers. (A) SET-2 cells were treated with low dose simvastatin (sim) or INCB018424 (424) alone, or in combination and were counted every two days using trypan blue. Combination treatment of sim and 424 lead to an exaggerated reduction in total viable cell numbers over time, in a dose-dependent manner. (B) Graph represents total viable cell fold reduction of drug treated samples compared to DMSO control at day 4. Experimental data represents a single experiment which was replicated in HEL cells (Figure 26), and multiple statins (Figure 28 & 29), with replicates supporting presented data.

Lovastatin cooperates with INCB018424 to enhance the reduction in viable HEL cells

We next wanted to determine if other statins had similar effects on JAK2-V617F-driven growth. We tested the ability of a second statin to cooperate with INCB018424. We found that lovastatin combined with INCB018424 also exaggerated the reduction in total viable HEL cell numbers (Figure 28A). 1 µM lovastatin led to a 1.6 fold reduction in total viable cell numbers, while 0.25 or 0.5 µM 424 led to a 2.8 or 5.9 fold reduction. Combining 1 µM lov with either 0.25 µM or 0.5 µM 424 resulted in an enhanced 5.6 or 7.5 fold reduction, respectively (Figure 28B).

Atorvastatin cooperates with INCB018424 to enhance the reduction in viable HEL cells

We investigated the potential cytotoxic effects of a third statin. Atorvastatin and INCB018424 combination treatment yielded an enhanced reduction in total viable cell numbers compared to single agent treatment (Figure 29). 1 µM atorvastatin led to a 2.3 fold reduction, while 0.25 µM 424 led to a 2.8 fold reduction in viable cell numbers at day 4. Combination treatment of 1 µM atorvastatin and 0.25 µM 424 resulted in a 5.4 fold reduction in viable cell numbers (Figure 29C).

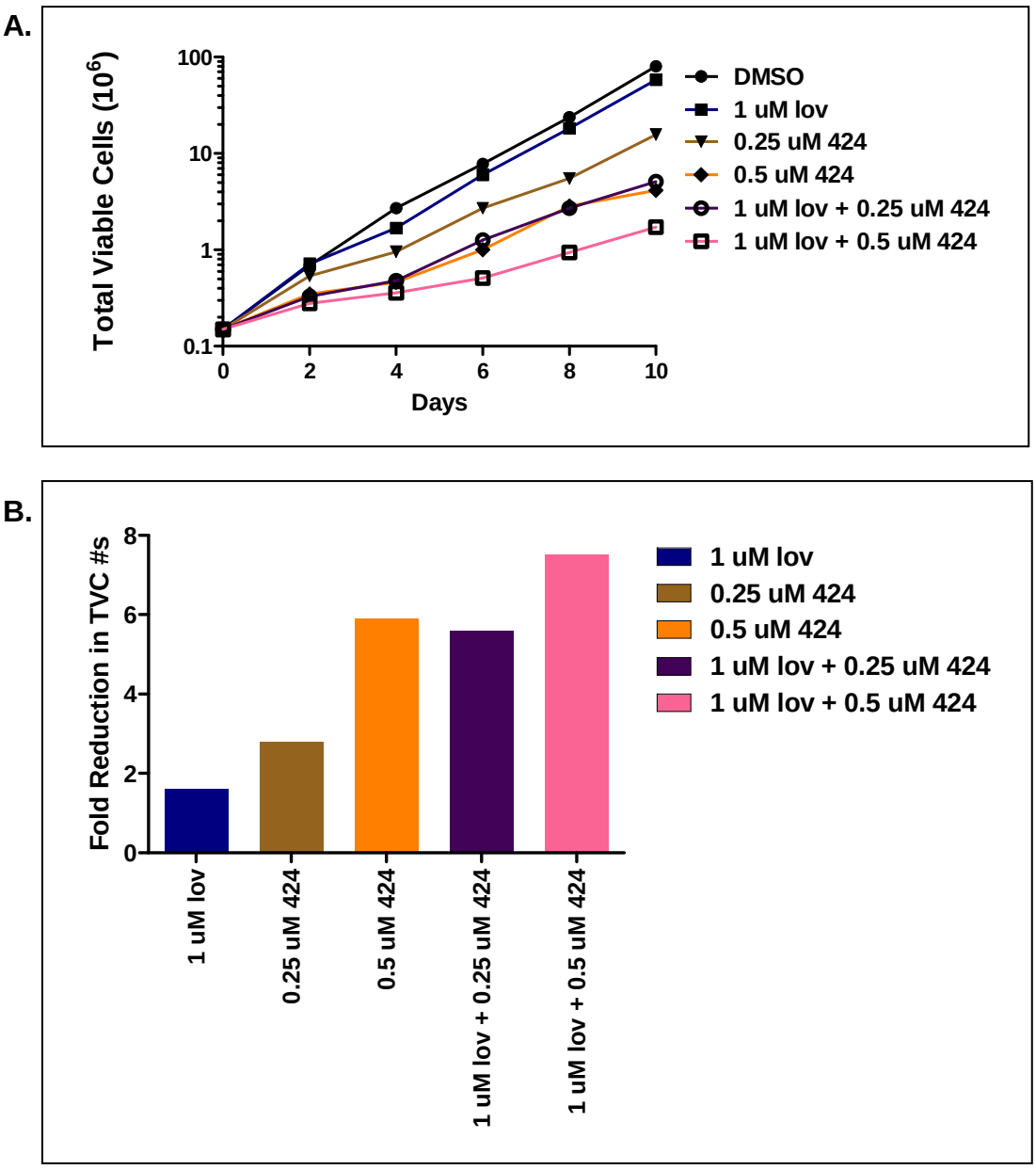


Figure 28: Lovastatin cooperates with INCB018424 in HEL cells to enhance the reduction in total viable cells. (A) HEL cells were treated with low dose lovastatin (lov) or INCB018424 (424) alone, or in combination and counted every two days using trypan blue. Lov combined with 424 resulted in an enhanced reduction in total viable cells. (B) Graph represents total viable cell fold reduction of drug treated samples compared to DMSO control at day 4. Experimental data represents a single experiment which was replicated with multiple statins (Figure 26 & 29), with replicates supporting presented data.

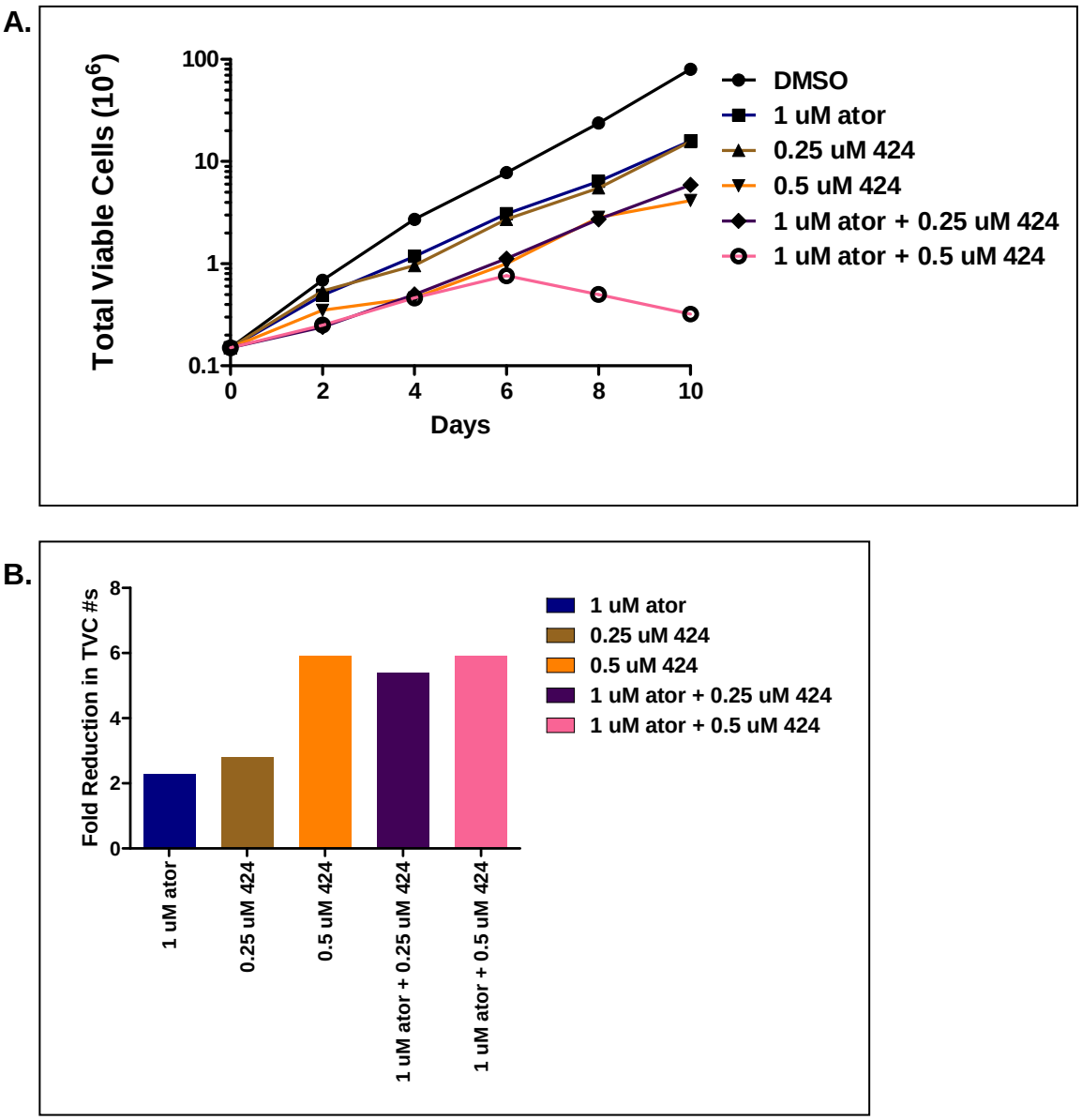


Figure 29: Atorvastatin cooperates with INCB018424 in HEL cells to enhance the reduction in total viable cell numbers. (A) HEL cells were treated with low dose atorvastatin (ator) or INCB018424 (424) alone, or in combination and counted every two days using trypan blue. Ator combined with 424 resulted in an enhanced reduction in total viable cells. (B) Graph represents total viable cell fold reduction of drug treated samples compared to DMSO control at day 4. Experimental data represents a single experiment which was replicated using multiple statins (Figure 26 & 28), with replicates supporting presented data.

Statins cooperate with INCB018424 to enhance apoptosis in JAK2-driven cells

To assess the effects of simvastatin and INCB018424 combination treatment on apoptosis, we analyzed the Annexin V-positive cell population by flow cytometry. HEL cells were treated with 1 and 5 µM simvastatin alone and in combination with 0.25 and 0.5 µM INCB018424 for 24 and 48 hours, and assessed for Annexin V binding. Combination treatment of simvastatin and INCB018424 gave rise to an even greater percentage of the cell population that was positive for Annexin V, compared to single agent treatment (Figure 30A). This is especially obvious for combination of 5 µM simvastatin and 0.5 µM INCB018424. Similar results were obtained with atorvastatin and INCB018424 treatment in HEL cells (Figure 30B).

Simvastatin combined with INCB018424 treatment induces a G1 arrest in JAK2-V617F-driven cells

To determine the effect of combination treatment with simvastatin and INCB018424 on the cell cycle in our MPN model cell lines, we stained cells with propidium iodide (P.I.) and analyzed DNA content by flow cytometry. We normalized the P.I. values (% of population) for each sample to the DMSO control by calculating the difference between the DMSO control and each sample to report the change in the % of cell population in each cell cycle phase (G1, G2, or S). After 24 hours of treatment we found a 4.22% increase in G2 after 5 µM simvastatin treatment. 424-treated cells displayed an increase of cells in G1 of 11.77% and 16.47% in the 0.25 and 0.5 µM 424-treated samples, respectively.

Combination treatment of 5 µM simvastatin with either 0.25 µM 424 or 0.5 µM 424 led to an additional increase in cells arrested in G1 (Figure 31), even though simvastatin alone did not induce G1 arrest.

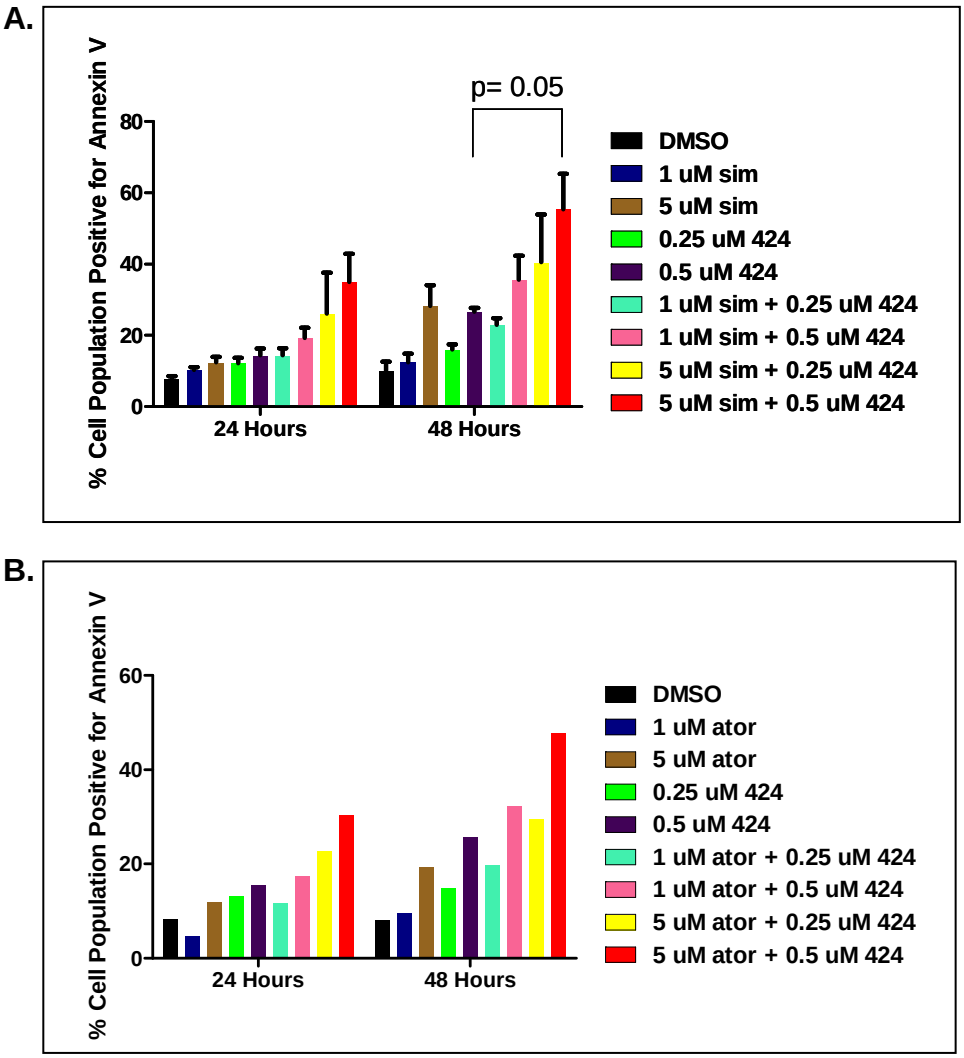


Figure 30: Statins cooperate with INCB018424 to enhance apoptosis in JAK2-V617F-dependent HEL cells. (A) HEL cells were treated with simvastatin (1 and 5 µM sim), or INCB018424 (0.25 and 0.5 µM 424) alone, or in combination for 24 or 48 hours and stained with Annexin V and analyzed by flow cytometry. Sim combined with 424 led to an enhanced induction of apoptosis in a dose and time-dependent manner. Error bars represent standard deviation of duplicate samples. (B) Combining atorvastatin (ator) with 424 after 24 or 48 hours led to an increase in Annexin V-positive HEL cells in a dose and time-dependent manner. Experimental data represents a single experiment which was replicated and preformed with multiple statins (Figure 30B), with replicates supporting presented data. P value was calculated using unpaired t-test using GraphPad Prism software.

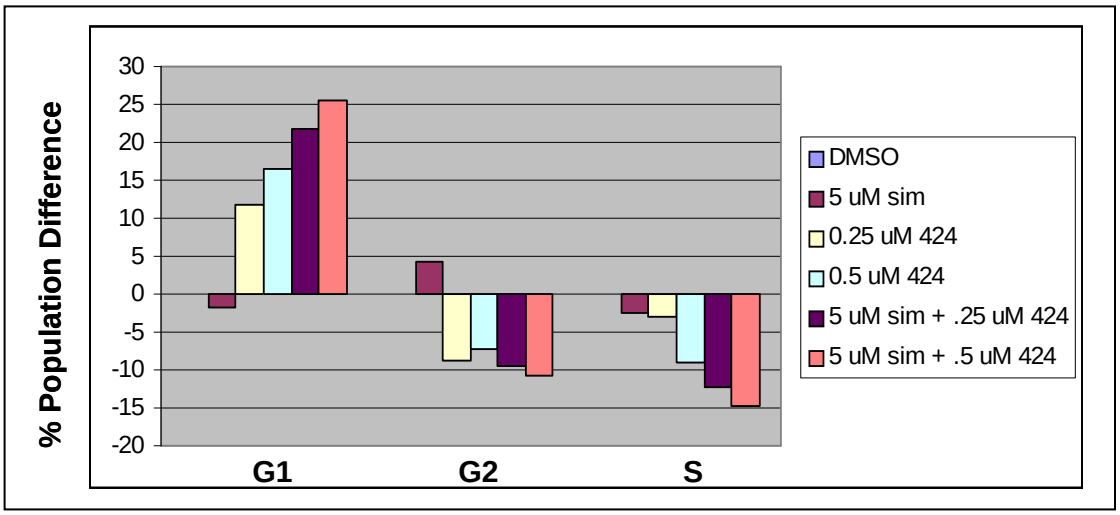


Figure 31: Simvastatin enhances the INCB018424-induced G1 cell cycle arrest. HEL cells were treated with DMSO, 5 µM simvastatin (sim), or 0.25 or 0.5 µM INCB018424 alone and in combination for 24 hours and stained with propidium iodide and analyzed by flow cytometry. Combination treatment led to a dose-dependent increase in cells arrested in G1. Experimental data represents a single experiment.

Bliss additivity model demonstrates cooperation between simvastatin and INCB018424

Next, we sought out to define the type of drug cooperation between statins and INCB018424 treatment in HEL cells. Specifically, we wanted to determine if the two drugs acted in an additive or synergistic manner. We assessed the cooperativity between simvastatin and INCB018424 in HEL cells after 72 hours of treatment using the Bliss additivity model [248]. We created a three-dimensional growth inhibition surface that was delineated by the single drug dose curves (Figure 32A). The Bliss additivity plane was calculated using the equation $I_{comb} = (IA + IB) – (IA * IB)$, where I_{comb} is the theoretical inhibition due to drugs A and B in combination and IA and IB are the observed inhibition due to each individual drug. We then calculated the 95% confidence interval and added

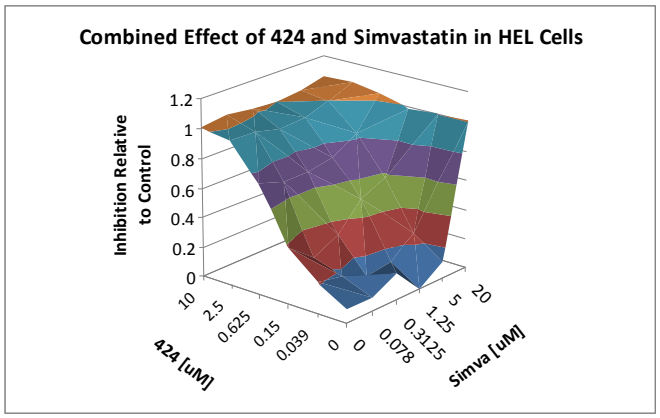
(upper limit) or subtracted (lower limit) this value from the theoretical plane to relay statistical significance to our observed values. Values above the upper 95% confidence interval are synergistic and a sum of these values found to be greater than 1 suggests moderate synergy between two drugs. The sum of our observed values (Figure 32B, upward cones) was found to be 0.43. Values below the lower 95% confidence interval are antagonistic and a sum of these values found to be less than -1 suggests moderate antagonism. The sum of these values (Figure 32C, downward cones) was found to be -0.32. All other data points are between these two planes and are considered additive.

The Bliss value of 0.43 suggests simvastatin cooperates with INCB018424 in an additive manner in HEL cells. However, we did identify potential doses and combinations that may be synergistic, as well as antagonistic using the lower 95% confidence interval (Figure 32C). We found that the combination of 1.250 µM simvastatin and 0.15 µM INCB018424 showed potential synergism, while 0.3125 µM simvastatin combined with 0.039 µM, 0.15 µM, or 0.625 µM demonstrated potential antagonism (Figure 32C).

Simvastatin reduces total viable Uke1 cells that persist in the presence of INCB018424

A recent report by Koppikar et al. has demonstrated that cells dependent on JAK2-V617F (Uke1 and SET-2 cell lines) can persist in the presence of

A.



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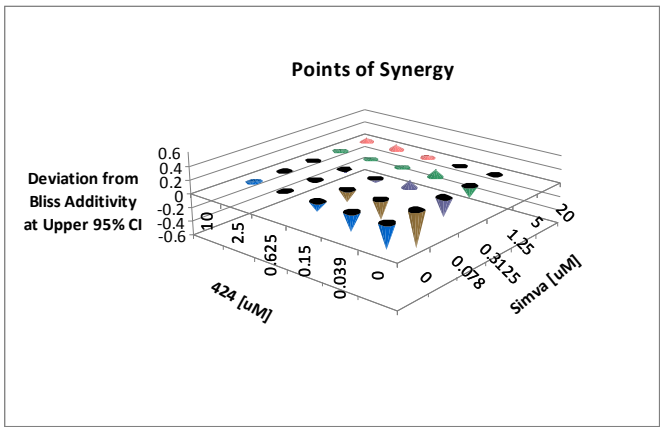

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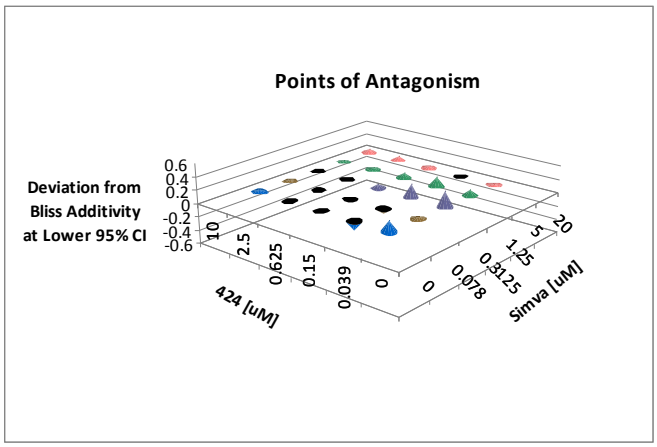

Figure 32: Simvastatin cooperates with INCB018424 in an additive manner. (A) HEL cells treated with 0 (DMSO) to 20 µM simvastatin (sim) alone, or in combination with 0 (DMSO) to 10 µM INCB018424 (424) for 72 hours were analyzed for cell viability using Promega CellTiter Glo luminescence. Graph plots growth inhibition of sim versus 424. (B) Bliss additivity model was used to determine Bliss values. The plane at 0 represents the upper 95% confidence interval. Upward cones represent statistically relevant synergistic values, while downward cones represent values below the 95% confidence interval. (C) Bliss values using lower 95% confidence interval. Downward cones represent statistically relevant antagonistic values, while upward cones represent values above the lower 95% confidence interval. Simvastatin cooperates with 424 in an additive manner, as suggested by the Bliss Value of 0.43. However, we identified doses with potential synergism at 1.25 µM sim and 0.15 µM 424. This experiment was done with assistance from Uwe and Lily Rix (Moffitt Cancer Center).

INCB018424 [249]. The term persistence is used rather than resistance because if the INCB018424 is removed from culture for a short period of time and re-introduced, the cells become sensitive to the drug again. We investigated the effect simvastatin would have on Uke1-Persistent (Uke1-P) viable cell numbers over time.We created Uke1-P cells by treating Uke1 cells with low doses of INCB018424 (0.1 µM) and selecting cells that grow out, subsequently increasing the doses to 1 µM until a population of cells grows in the presence of 1 µM 424. Sim (5 µM) prevented the outgrowth if Uke-P cells over the course of the 8 day experiment (Figure 33).

Discussion

It is accepted in the MPN field that activation of JAK/STAT signaling is a key feature among all classical MPN patients [65]. Inhibitors that target JAK enzymes have recently been approved to treat MF patients because of the improved effects on splenomegaly and constitutional symptoms [94, 205], suggesting benefit in targeting JAKs in patients. Additionally, the COMFORT-1 and 2 JAK inhibitor clinical trials demonstrated that regardless of presence of the JAK2-V617F mutant, patients responded similarly to ruxolitinib (INCB018424) [29, 93-95], suggesting again that JAK activity is important in MPNs. More recently, we were the first to report that statins alone induce cytotoxic effects in JAK2-V617F-dependent cell lines and primary MPN cells (Chapter 2) [238]. In our current study, we sought out to determine if JAK inhibitor therapy cooperated with statin therapy in JAK2-V617F-dependent cells to ultimately identify

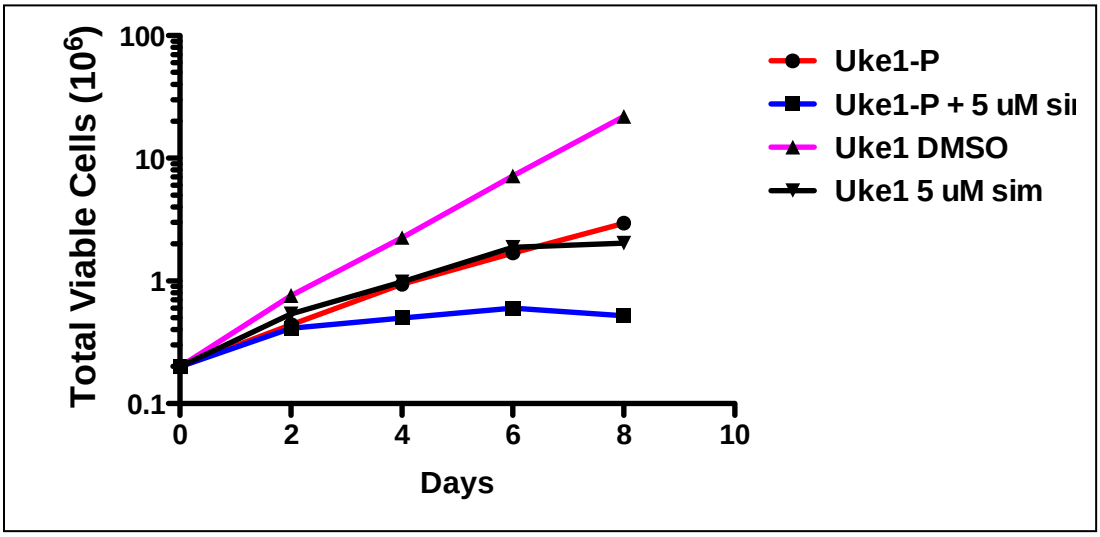

Figure 33: Simvastatin reduces total viable Uke1 cells that persist in the presence of INCB018424. Uke1 cells were made persistent to 1 µM INCB018424 (424) by treating Uke1 cells with low doses (starting dose 0.1 µM 424), selecting for cells that grew out, and increasing the concentration of 424 up to 1 µM. Cells were then treated with 5 µM simvastatin (sim) for 8 days and counted using trypan blue. 5 µM sim prevented the outgrowth of Uke-P cells growing in the presence of 1 µM 424. Experimental data represents a single experiment which was replicated three times, with replicates supporting presented data.

additional therapeutic strategies for MPN patients. Indeed, we now provide evidence of the cooperation between statins and JAK inhibitors.

Our studies showing that multiple statins cooperate with multiple JAK inhibitors in multiple JAK2-V617F-dependent cell lines are novel. We demonstrate that statins cooperate with JAK inhibitors to enhance the reduction in total viable cell numbers and viability compared to the single agents alone (Figures 1-6). These data are relevant and intriguing for the current MPN field. First, the results from the two phase 3 clinical trials involving INCB018424 (ruxolitinib), COMFORT-1 and COMFORT-2, did show that some patients did better than others and it is still poorly understood why that is the case. A key finding from the clinical trials with JAK2 inhibitors is that these inhibitors do not

decrease allele burden [94, 205]. That is, they do not get rid of the neoplastic MPN cells in patients. Therefore, an approach that enhances the efficacy of MPN cell death in response to therapy is still needed in order to induce remission in MPN patients. Second, it has been proposed that combination therapies of JAK2 inhibitors and other agents may play a major role in future MPN therapies [116-117]. For example, studies have shown cooperation between JAK2 inhibition and Hsp90 inhibitors as well as HDAC inhibitors on MPN cells. We believe our studies suggest that statins may be another option for combinatorial therapeutics with JAK2 inhibitors.

Importantly, we showed in two separate cases, with two separate JAK inhibitors that statins are still effective at inducing cytotoxic effects in our JAK2-V617F-driven cells that show signs of resistance. First, we show that after eight days of treatment, HEL cells become resistant to the pan JAK inhibitor, JAK Inhibitor I, Simvastatin treatment blocked the ability of the HEL cells treated with JAK Inhibitor I to acquire resistance (Figure 24A). Second, we showed that low dose simvastatin (5 μM) was effective at inhibiting the growth of Uke1-persistent cells. These cells persistently grow in the presence of INCB018424, suggesting that resistance or persistence to the JAK inhibitor does not also provide resistance to the cytotoxic effects of simvastatin (Figure 33). Since some MPN patients are resistant to the current standard of care (ex. Hydroxyurea) [29, 93, 95], we question if statins can provide therapeutic benefit in these cases. We propose statins may provide benefit in MF or other MPN patients resistant or

persistent to therapy, contributing to the management of the high peripheral cell burden.

Further investigations are underway to determine the effect of combining statin and JAK inhibitor on MPN cells. In collaboration with Ross Levine (Memorial Sloan Kettering Cancer Center) we are utilizing an MPN mouse model to determine if statins alone or in combination with JAK2 inhibition can inhibit MPN formation *in vivo*. We will also be performing colony formation assays with primary mononuclear cells from JAK2-V617F-positive MPN patients treated with statins combined with 424.

Our assessment of the type of cooperation exhibited between statins and INCB018424 demonstrated an additive cooperation. We did identify potential dose combinations of statins (1.25 µM simvastatin combined with 0.15 µM INCB018424) that may be synergistic, and we are currently investigating this prospect further. It is important to consider that the Bliss cooperation analyses, are short-term experiments, 72-96 hours, whereas the growth curve experiments run between 192-264 hours and is where we can observe a significant effect of statins. It will be interesting to determine if differences in the length of time of experiments affects synergy designation.

Our studies described in this chapter are the first to demonstrate the cooperation between JAK inhibitors and statins in JAK2-V617F-dependent cells. We show that multiple statins cooperate with multiple JAK inhibitors in multiple JAK2-V617F-dependent cells. We propose that statins combined with JAK inhibitor therapy in patients may lead to an improved response compared to JAK

inhibitor therapy alone. Statins may provide an affordable, safe therapeutic addition to JAK-targeted therapy for MPN patients.

Materials and Methods

Growth curves

HEL, SET-2, Uke-1, or Uke1-P cells were plated at a concentration of 0.1 - 0.25 X 10^6 cells/mL and treated with 0 (dimethyl sulfoxide (DMSO)), 1 or 5 µM simvastatin (Sigma-Aldrich #S6196), lovastatin (Sigma-Aldrich #M2147), atorvastatin (Sigma-Aldrich #PZ0001), 1 µM JAK Inhibitor I (Calbiochem #420097), or 0.25 - 0.5 µM INCB018424 (Chemietek) alone, or in combination, maintaining the DMSO content to 0.1% across all samples. Treated cells were counted every two days with trypan blue using a hemocytometer. Cells were split every two days, refreshing drugs and media.

Annexin V staining

HEL cells were treated with 0 (DMSO), 1 or 5 µM simvastatin or atorvastatin, alone, or in combination with 0.25 - 0.5 µM INCB018424 (Chemietek) for 24 or 48 hours. 1 X 10^6 cells were washed with 1X PBS (room temperature (RT)) and reconstituted in 100 µL of 5% BSA. 0.5 X 10^6 cells (50 µL) were added to 50 µL 2X binding buffer (BD Biosciences), 8 µL [10 µg/mL] propidium iodide (P.I.) (BD Biosciences), and 3 µL Annexin V-FITC (BD Biosciences #556570), and incubated for 15 minutes at RT in the dark. 300 µL of

1X binding buffer (BD Biosciences) were added and stained cells were analyzed by flow cytometry (Calibur 2).

Cell cycle analysis

1 X 10^6 HEL cells were treated with 0 (DMSO), 1 or 5 µM simvastatin or atorvastatin, alone, or in combination with 0.25 – 0.5 µM INCB018424 (Chemietek) for 24 or 48 hours. Cells were washed with 1X, RT PBS and resuspended in 1X PBS. 900 µL of ice-cold 70% ethanol was added to 100 µL of cells in PBS in a drop-wise fashion and vortexed on low speed. Cells were incubated overnight at -20º C. After overnight incubation, samples were thawed on ice and subsequently centrifuged at 14.5 X 10^5 X g for 60 seconds. Cells were washed with 1X cold PBS and resuspended in 500 µL of (0.1% Triton-X-100-PBS + 10 µg/mL P.I. + 1 mg RNase A). Incubated sample in the dark for 30 minutes at RT and analyzed by flow cytometry.

Bliss cooperation

HEL cells were treated with 0 (DMSO) to 20 µM simvastatin (Sigma Aldrich #S6196) or 0 (DMSO) to 10 µM INCB018424 (Chemietek), alone or in combination, in 384 well dishes at a plating confluency of 0.1 X 10^5 cells/mL. Treatment incubations included 24, 48, and 72 hours at 37º C and 5% CO_2. DMSO content was maintained at 0.4% across all samples. Cell viability was determined using Promega CellTiter Glo luminescence (Promega #G7571).

Synergy was determined using % viability inhibition values and calculated using the Bliss addivitity model [248].

Chapter 5

Summary, Final Discussion, and Future Work

Summary and Discussion

Our work has provided multiple novel discoveries for the MPN field. First, JAK2-V617F, a key etiologic factor for MPN development, is localized to lipid rafts (Chapter 2). Second, JAK2-V617F signaling is dependent on lipid rafts (Chapter 2). Third, MPN cells dependent on JAK2-V617F are sensitive to statin treatment (Chapters 2 and 4). Fourth, statins cooperate with JAK2 inhibitors to inhibit the growth and induce apoptosis of JAK2-V617F dependent cells (Chapter 4).

We have demonstrated that the localization of the JAK2-V617F mutant to cholesterol-rich lipid rafts may be important for the full transforming potential of this oncogene. Disruption of lipid rafts with agents that alter membrane cholesterol reduced the localization of JAK2-V617F protein in lipid rafts (Figure 8 and 9). We found that multiple cholesterol alteration agents are effective at downregulating JAK2/STAT signaling induced by JAK2-V617F (Figures 10A and 10C, respectively).

Currently, there are no lipid raft disrupting agents on the market for use in humans, so to investigate functional aspects of targeting lipid rafts in JAK2-V617F-driven MPN cells, we utilized the statin class of drugs. The JAK2/STAT

pathway is accepted as being crucial to MPNs and is therefore an appreciated target for therapeutic intervention [65]. We hypothesized that cholesterol-lowering statins may disrupt lipid rafts and hence, disrupt the JAK2-V617F signaling complex at the membrane, ultimately attenuating JAK2/STAT signaling. Indeed, cholesterol lowering statins downregulated JAK2/STAT signaling, inhibited cell growth, and induced apoptosis in JAK2-V617F-dependent cells (Figure 10, 11A/B, and 12A/B, respectively). Very importantly, the cytotoxic effect of statins was not only seen in MPN model cell lines, but primary JAK2-V617F-positive MPN cells were also sensitive to low dose statin treatment.

Additionally, since JAK2 inhibitors alone are ineffective at reducing the allele burden in MPN patients and thus fail to induce remission, the cooperativity between statins and JAK2 inhibitors we have observed is highly relevant to a need in the MPN field, that is, enhanced therapeutic killing of MPN cells. Combination treatment with statins may provide opportunities to sensitize MPN cells to JAK2-targeted therapy. This additional treatment, whether it is directed at JAK2 signaling or not, may also decrease the development of de novo or innate resistance that has been seen in MPN patients. Such a combination treatment may also decrease the rate at which MPN patients transform to AML.

An important aspect to our work involves the dosage used in our experiments. Our *in vitro* experiments are within physiological range that could be tolerated in humans. Studies surrounding lovastatin showed that patients can tolerate ~3.9 µM without any toxicities long-term [250]. It has also been shown that patients given higher concentrations, ~12 µM for short time points (7 days) in

cycles show no toxicity complications [250]. Another strategy has been employed to avoid toxicity in patients given high doses of statins, whereby the cholesterol precursor, squalene is supplemented, allowing patients to tolerate higher statin doses [251]. Further investigation is needed to determine any implications of therapeutic benefit with chronic concentrations such as these in MPNs.

In summary, we hypothesize that statins promote a cytotoxic effect in MPN cells, and propose that statins, as a mono-therapy or more provocatively in a combinatorial approach with JAK2 inhibitors, may provide an effective therapeutic approach to improve the lives of MPN patients (Figure 34).

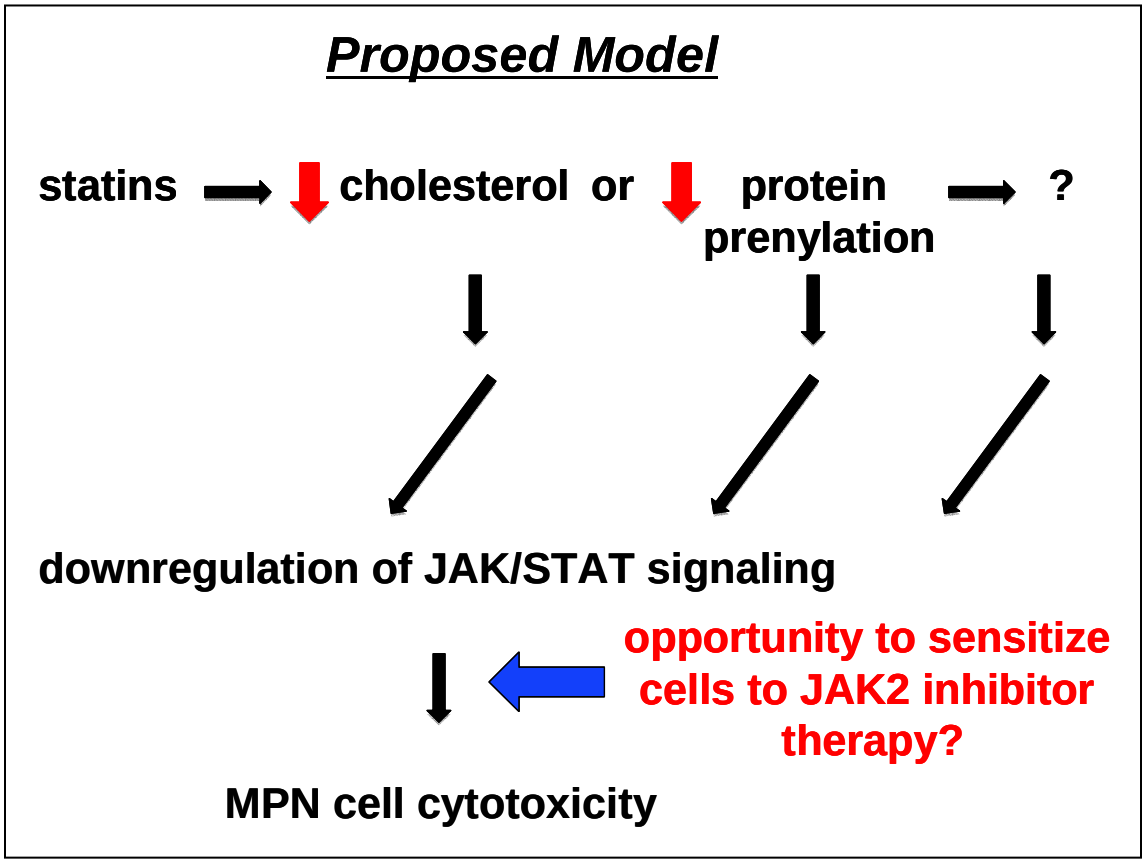

Figure 34: Proposed model for targeting the mevalonate pathway with statins in conjunction with JAK2 inhibitors in MPNs.

Future Work

We believe the work presented in this dissertation encourages additional studies focused on investigating the potential role of lipid rafts in JAK2 signaling in MPNs and the use of statins in novel therapeutic strategies for MPNs. We intend to further investigate the role of lipid rafts in signaling driven by mutations other than JAK2-V617F. Mutations of particular interest include *JAK2 exon 12*, *MPL*, and *LNK* mutations. In addition, we would like to address the role of cytokine receptors to further understand the sensitivity of JAK2-V617F signaling to lipid raft alteration. For example, we are interested in comparing the sensitivity to lipid raft disruption in the setting of EpoR/JAK2-V167F (important in PV) versus MPL/JAK2-V617F (important in ET) versus GCSF-R/JAK2-V617F (important in MF). This is of particular interest because the JAK2-V617F mutant requires the expression of a cytokine receptor and these receptors may play important roles in MPNs. We will also analyze the effects of statins alone, and in combination with JAK2 inhibitors in JAK2-V617F-driven MPN murine models. In addition, we will expand our studies with primary patient samples to determine the extent to which statins cooperate with JAK2 inhibition in both JAK2-V617F-positive and negative patient cells. With encouraging results from these and other experiments, a clinical trial addressing statins in MPN therapy will be a possibility in the near future.

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