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# Allosteric modulation of VLA-4 with the atypical antipsychotic thioridazine

Bart Williams

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# Allosteric Modulation of VLA-4 with the Atypical Antipsychotic Thioridazine

by

**Bart Williams**

B.S., Biopsychology, University of California  
Santa Barbara, 2004

THESIS

Submitted in Partial Fulfillment of the  
Requirements for the Degree of

Master of Science  
Biomedical Sciences

The University of New Mexico

Albuquerque, New Mexico

August, 2011

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## **Abstract**

A recent primary drug screen by the University of New Mexico Center for Molecular Discovery uncovered a novel group of allosteric-modulators of VLA-4 integrins known as phenothiazines. Phenothiazine family compounds were initially discovered as organic dyes in the 1800's, the most well-known of which is methylene blue. Relatively soon thereafter phenothiazines were found to be useful clinically, a trend that has continued in the century since peaking as antipsychotics in the 1950's. Today, phenothiazines are still being pursued clinically as seen in a number of recent patents for phenothiazine use in autoimmune disorders. This study sought to further characterize the newly discovered relationship between phenothiazines and VLA-4 integrin in a stepwise progression from *in vitro* experiments to an *in vivo* animal model. Here, it is shown that the phenothiazine compound thioridazine causes broad inhibition of adhesion related activity including: the rapid interruption of VLA-4 bound fluorescent

molecules, the disaggregation of VLA-4 and VCAM-1 attached cells, rapid reduction in intracellular calcium signaling, and loss of cellular motility to  $G\alpha_i$  chemokines. Furthermore, it is shown that thioridazine treatment can cause a 3-fold increase in mobilization of murine hematopoietic stem and progenitor cells. Combined, these data answer forty year old questions about the nature of phenothiazine treatment, provide insight in to the mechanism of action and provide evidence for the potential of VLA-4 allosteric modulators *in vivo*.



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# 1 Introduction

Integrins are highly expressed adhesion receptors, critical to microscopic processes such as cell growth, maturation, differentiation and macroscopic processes such as embryonic development, immune response, and tissue repair. At first glance is a seemingly straight forward appearance which dissembles their complexity and function (Figure 1). Crystallography has revealed integrin structure as comprised of two N-terminus ligand binding domains each extended  $\approx 170\text{\AA}$  extracellularly by "leg" domains which span down through the cell membrane into the cytoplasm (Arnaout et al., 2005; Luo et al., 2007). The "leg" structures are made of

multiple domains which together can collapse, bend, and twist creating multiple conformations altering both extracellular adhesion and intracellular signaling. Adding to this complexity, the combined "head" and "leg" domains make-up dis-

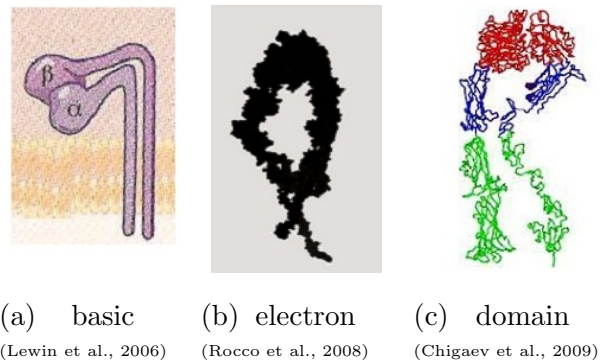


Figure 1: **Integrin Structures**

tinct  $\alpha$  and  $\beta$  subunits. The paired subunits form a multitude of integrins for cell specific expression, loss of functionality redundancy, and other specialized roles. Currently, there are 24 known  $\alpha\beta$  integrin combinations formed by 18 possible  $\alpha$  and 8 possible  $\beta$  subunits. This study focuses on the  $\alpha4\beta1$ -integrin (Very Late Antigen-4, VLA-4) one of many integrins types expressed on hematopoietic cells including mature leukocytes, progenitors, and cancer cell types (Lewin et al., 2006). VLA-4 is crucial to regulating the hematopoietic populations, retaining progenitors in

the bone marrow for re-population, and in trafficking leukocytes during immune responses (Figure 2). With such important roles in normal physiology VLA-4 is also involved in the pathogenesis of a number of diseases. In fact, a number of compounds directly targeting the VLA-4 ligand binding pocket have already been developed and researched in therapies including, stem cell mobilization, cancer treatment, and autoimmune diseases. Moderate success with these compounds in animal trials, clinical trials and even FDA approval for some compounds suggests that VLA-4 and other integrins represent viable therapeutic targets.

Unfortunately, the direct VLA-4 antibodies and small molecule compounds developed to date may not represent the best possible therapeutic options (Cox et al., 2010). Previous work has demonstrated compounds binding directly to the VLA-4 ligand pocket can displace native ligand but in doing so still retain the integrin in an activated conformational state (Billard and McIntyre, 2008; Njus et al., 2009). The retention of the activated conformation by these compounds maintains intracellular signaling capabilities and thus potentially unwanted effects during treatment. In cancer, for example, static VLA-4 activation is thought to provide growth signals and resilience against chemotherapy. Efficacy of these direct compounds can also be affected by different signaling environments within the body. In areas rich with chemokine and growth factor expression, such as the bone marrow, indirect signaling alters the affinity with which integrins bind to ligand. Thus, a need exists to find novel VLA-4 antagonists which could potentially improve therapeutic efficacy over the current direct VLA-4 antibody and small molecule compounds.

In 2008, a high-throughput flow cytometry screen designed by Larry Sklar and Alexander Chigaev was conducted to discover allosteric modulators of VLA-4 (Sklar and Chigaev, 2008). Among the active compounds were a group of phenoth-

iazines, a family of drugs with a long history in medicine, known primarily as early-antipsychotics. This finding may have been the first to demonstrate a connection between phenothiazines and integrins. Clinical use of phenothiazines for over a cen-

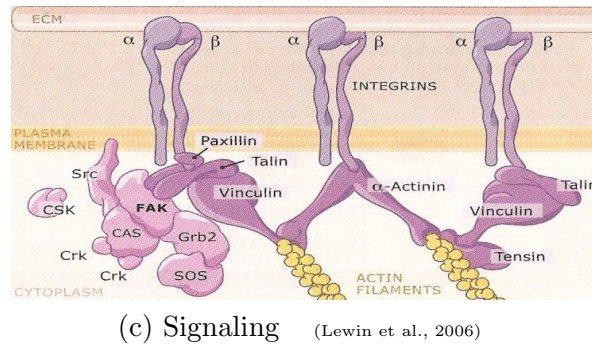
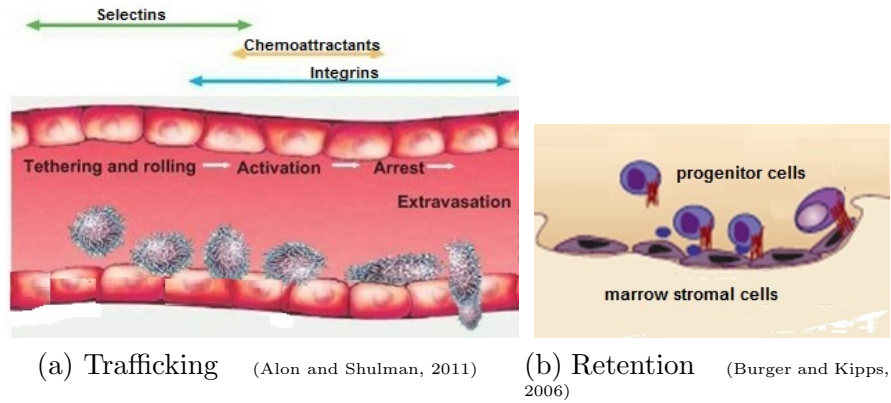


Figure 2: **VLA-4 Activities:** (a) Illustrates VLA-4 mediated leukocyte capture and trafficking within the bloodstream. Capture starts with selectins, a class of adhesion receptors which cause leukocytes to roll along the lumen of the endothelium. Next, contact with surface bound chemoattractant molecules along the endothelial lumen activates VLA-4 integrins causing firm adhesion. The arrested leukocytes then crawl and extravasate out of the bloodstream. (b) Illustrates VLA-4 mediated homing and static adhesion of hematopoietic progenitors within the bone marrow. Chemoattractants expressed by marrow stromal cells activate VLA-4 integrins on the progenitor cells mediating attachment. Static adhesion also occurs creating long term binding that holds progenitors in the marrow.(c) Illustrates the various outside-in signaling pathways which are coupled to VLA-4 receptors. VLA-4 adhesion causes conformational changes in the cytoplasmic subunit tails initiating scaffolding of several signaling and structural proteins. The illustration also demonstrates the capacity for multiple integrins to consolidate on the cell surface and expand the scaffold.

tury does however suggest VLA-4 related mechanisms in a number of old case studies and more recent patents which identify therapeutic potential in cancer, asthma, and rheumatoid arthritis treatment. Combined the findings from the screen and past literature suggested phenothiazines could allosterically regulate VLA-4 both *in vitro* and *in vivo*. Therefore, it was the intent here to study a phenothiazine compound from the 2008 screen in a number of *in vitro* and *in vivo* experiments which had previously only tested direct VLA-4 compounds. It was believed that the chosen phenothiazine would lead to similar but potentially accelerated and more complete responses as an allosteric modulator of VLA-4 compared to direct compounds.

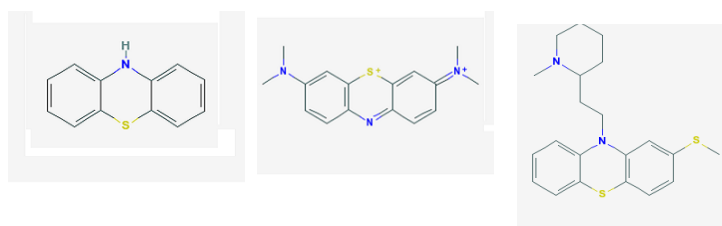
## 1.1 Phenothiazine History

Phenothiazine compounds were first synthesized in the late 1800's (Mitchell, 2006). Tricyclic structures containing a central thiazine ring, early phenothiazines such as methylene blue were first developed as colourants or dyes (Figure 3). In the following

decades it was discovered that methylene blue could not only bind biological tissue but could be used

therapeutically as an antimalarial, antipsychotic,

and analgesic. The prescribed use of phenothiazines for psychiatric disorders has been widespread in the century since. At least sixty unique phenothiazine compounds with different substitutions were developed for clinical practice (Jones, 1996). Such expansion and use led to the discovery and rediscovery of many therapeutic



(a) Phenothiazine (b) Methylene blue (c) Thioridazine

Figure 3: **Phenothiazine Structures.**

purposes for phenothiazines including; immunomodulatory, chemotherapeutic, antipruritic, antiemetic, antifungal, antibacterial, antihelminic, and antimalarial. The broad range of biological activity suggests numerous possible targets and mechanisms of action. Based on measurable increases in metabolite concentrations, early clinical efficacy was correlated to the blockade of neurotransmitter receptors, namely dopamine and serotonin (Sudeshna and Parimal, 2010). A later study demonstrated that various side groups altered muscarinic receptor affinity and correlated inversely with extra-pyramidal side effects (Snyder et al., 1974). Recent reviews indicate that the specific phenothiazine target of action is not clear. The authors consider and discuss the the multiple enzymatic, redox, and signaling effects which can all vary depending on cell type and the numerous phenothiazine substituents (Mitchell, 2006; Sudeshna and Parimal, 2010; Kinross-Wright, 1967) Thus, phenothiazines have a well-established history of clinical efficacy yet have a number of paradoxes concerning their target site and mechanism of action.

The phenothiazine family's long existence and wide ranging therapeutic efficacy has led to a number of observational and case studies documenting unexpected clinical effects. Some of these studies indicate a potential connection to integrin modulation. In one group of studies, multiple investigators reported atypical lymphocyte presence in the peripheral blood of phenothiazine treated patients (Fieve et al., 1966). In one of these studies, phenothiazine treated schizophrenic and non-schizophrenic patients averaged a  $\approx 12\%$  increase in atypical lymphocytes over non-phenothiazine treated patients. When this research was conducted in 1966, with hematopoiesis not yet fully understood, the atypical lymphocytes were described as dividing and differentiating lymphocytes which may have "escaped" into the blood. The pictures and descriptions from this research indicate that atypical lymphocytes are what we

know today as hematopoietic progenitor cells (HSC's) escaped from spleen or bone marrow. Thus, these studies suggest that phenothiazines may somehow alter HSC homing or mobilization but the mechanism of action is unclear.

A second set of case studies have implicated phenothiazines in the treatment of cancer. These studies detail miraculous recoveries in patients whom were provided phenothiazines as antidepressants or antiemetics following chemotherapy (Jones, 1996). Marked improvements were seen in numerous types of cancer; bronchial, breast, squamous cell, even in cases where chemotherapeutic resistance had set in. These case studies have led to a number of *in vitro* experiments showing that phenothiazines alone or synergistically with anticancer drugs cause necrosis and apoptosis in cancer cell lines (Zhelev et al., 2004; Lialiaris et al., 2009; Darkin et al., 1984). More recently phenothiazine efficacy has been expanded in a number of patents claiming phenothiazines as serotonin receptor antagonists to treat immunopathologies including, asthma, rheumatoid arthritis, and other inflammatory diseases (Roth et al., 2005, 2009; Roth and Zope, 2009; Urbahns et al., 1999). While these studies mention other potential targets rather than integrins, the modulation of VLA-4 has been linked to all of these pathologies. Thus, decades of expanding clinical use indicates numerous potential therapies with phenothiazines where VLA-4 allosteric modulation may potentially contribute to the mechanism of action.

## **1.2 VLA-4 *in vitro***

*in vitro* studies have identified three independent conformational features to describe VLA-4 integrins; mechanical form (bent or extended), affinity state of the ligand pocket, and occupancy of the binding pocket (Chigaev et al., 2009). The sub-



classifications, bent or extended, high affinity or low affinity, bound or unbound, can be combined and arranged to postulate eight different physiological conformations. Different *in vitro* methods have been used to study each feature; florescent resonance energy transfer experiments to characterize mechanical form, flow cytometry experiments to characterize affinity and ligand occupancy, and shear flow experiments to characterize adhesion (Kim et al., 2003; Alon and Ley, 2008; Chigaev et al., 2001). Combined these methods have established how the conformations are regulated both independently and together. In the cases of affinity and extension both have been shown to be controlled by inside out signaling. While each can be regulated independently G-protein coupled receptor signaling cascades have been shown to cause both rapid unbending and increased affinity (Chigaev et al., 2007). This type of signaling can be initiated *in vitro* with chemokines, such as SDF-1 $\alpha$  and fMLF, acting through their respective G $\alpha_i$  receptors CXCR4 and FPR, to mediate contact events including trafficking, rolling, tethering and firm adhesion. The third conformational feature, a structural change induced by binding pocket occupancy is highly independent of both affinity, extension and G-protein coupled inside-out signaling. Understanding the independence of this conformational change was the basis for the 2008 screen which identified the phenothiazine family.

The screen used the probe HUTS-21, which conditionally binds VLA-4 when ligand occupied, to indirectly monitor occupancy of the binding pocket. HUTS-21 is a fluorescent antibody which targets a ligand induced binding site (LIBS) on the hybrid domain of  $\beta 1$  subunit. Prior to the screen, *in vitro* experiments determined binding of HUTS-21 was directly correlated with ligand occupancy and uncorrelated to inside-out signaling (Chigaev et al., 2009). This initially led to an effort to reclassify existing VLA-4 compounds as either agonists or antagonists and as direct or indirect

modulators. The subsequent experiments indicated that many compounds known to modulate VLA-4 were in fact direct competitors to the ligand binding pocket and exposed the LIBS to HUTS-21 (Njus et al., 2009). The screen was thus designed to address the need for novel compounds which could obscure the LIBS. The results showed that phenothiazine family compounds could reduce the fluorescent signal generated by HUTS-21 binding to the VLA-4 LIBS, thus creating a need for further studies to establish the ability of phenothiazines to modulate VLA-4 adhesion.

### 1.3 VLA-4 Pathologies

The broad expression of VLA-4 across different cell populations indicates its importance in human systems. Loss of  $\beta 1$  or  $\alpha 4$  are both embryonic lethal in knockout mice (Imai et al., 2010; Lewin et al., 2006). In keratinocytes conditional  $\beta 1$  knockout mice showed deficits in basal lamina assembly resulting in alopecia and skin blistering. Other conditional knockouts aimed at hematopoietic cells showed altered HSC homing, loss of differentiation, and increased progenitor populations in the spleen and peripheral blood. Counter to VLA-4 reduction, over-expression and/or over-activation of VLA-4 has been linked to the inflammation responsible for autoimmune pathogenesis (Jackson, 2002). Inflammation is often the result of abnormal leukocyte recruitment and accumulation at affected organs and tissues. Both the recruitment and accumulation are thought to be largely VLA-4 and integrin mediated. A VLA-4 drug, natalizumab, an  $\alpha 4$  antibody, was in fact FDA approved in 2004 to treat two autoimmune diseases, multiple sclerosis and Crohn's disease (Johnson, 2007). In multiple sclerosis natalizumab is thought to block VLA-4 mediated tethering and extravasation of leukocytes across endothelial cells at the blood brain barrier decreasing

inflammation and subsequent myelin loss. Unfortunately while promising in clinical trials at lowering the incidence of lesions in both Crohn's and multiple sclerosis, side effects, namely the development of PML (progressive multifocal leukoencephalopathy) in a few patients has led to restricted guidelines for use. Development of PML is typically related immunosuppression allowing a latent virus to attack the brain. Concern over immunosuppression has halted a number of other integrin antibodies in clinical trials as well including valategrast, an orally delivered VLA-4 antagonist showing promise in an asthma trial (Woodside and Vanderslice, 2008). Regardless, these integrin targeted drugs such as natalizumab along with the knockout studies have therapeutically established VLA-4 in autoimmune pathogenesis. Thus, there is a clinically established relationship between VLA-4 and autoimmune diseases, phenothiazines being patented for clinical therapy in the same diseases, and *in vitro* evidence of phenothiazines modulating HUTS-21 antibody from the  $\beta 1$  subunit of VLA-4.

## 1.4 Mobilization

While, both integrin and phenothiazine based treatments are being actively pursued for autoimmune disorders, hematopoietic stem cell (HSC) mobilization is an area where only VLA-4 small molecule drugs are being tested. The HSC population is made up of several groups of varying long term and short term undifferentiated cells and the early progenitors for each blood cell lineage (Challen et al., 2009). These progenitor populations are critical and must be replenished and maintained throughout life to facilitate hematopoiesis. The niche and center for this HSC growth is the bone marrow. Under normal conditions these early progenitor and undifferentiated

stem cell populations can also be found circulating in the peripheral blood. The relative numbers in the periphery are low but fluctuate with steady-state homeostatic processes including circadian rhythm, injury, thymus re-population and marrow overpopulation (Lane et al., 2009; Lucas et al., 2008). The major mechanisms regulating the release and homing of these cells include maturation based receptors such as CD34, chemokines such as stem cell factor and integrins. Clinically, acquisition of these early undifferentiated HSCs has become an essential step in transplantation therapies to ensure successful engraftment. Pharmaceutical induced mobilization is now preferred over direct bone marrow harvesting due to higher undifferentiated stem cell yields, faster hematopoietic repopulation, and technical simplicity. These treatment goals along with research of the HSC population have led to a number of potential compounds for mobilization. The three major classes of compounds currently in use are granulocyte colony stimulating factor (G-CSF), AMD3100/3465, and VLA-4 small molecules.

G-CSF is a growth factor and the predominate choice for HSC mobilization. It is administered on consecutive days in order to first induce a large increase in the HSC population (Wagers et al., 2002; Broxmeyer et al., 2005). On day four to five of treatment the overgrown HSC population releases from the bone marrow into the periphery creating 1000-fold increases in the circulating HSC population. Such expansion of the relatively small stem cell population makes G-CSF a very advantageous mobilizing agent. However, wide patient variability along with comparison studies which have shown physically harvested marrow to be better for long term hematopoietic repopulation have raised concerns about G-CSF. A group from Stanford University lead by Irving Weissman believed expression of adhesion molecules were likely altered in G-CSF mobilization and causing the issues with repopulation

(Wagers et al., 2002). They measured expression of multiple  $\alpha$ -integrin subunits as well as  $\beta 1$  on isolated bone marrow and peripheral blood stem cells following 0, 2 and 4 days of G-CSF stimulation. They found reduced  $\alpha 4$  and  $\beta 1$  expression after 4-day G-CSF stimulation, and concluded the decrease in integrin expression in G-CSF mobilized cells was associated with the reduced repopulation capacity. Thus, while G-CSF mobilization is largely based on population expansion there is evidence that VLA-4 expression is also involved in HSC mobilization homing capacity.

AMD compounds were originally developed as targets of the HIV co-receptor CXCR4. The bicyclam AMD3100 (Plerixafor) was withdrawn as a candidate in HIV therapy but has since become known as a CXCR4 chemokine receptor antagonist (Broxmeyer et al., 2005). The CXCR4 receptor is broadly expressed across hematopoietic cells, including HSC's. It is coupled to  $G\alpha_i$  which produces both calcium and PI3K signals in response to its predominant ligand, stromal cell-derived factor-1 (SDF-1, CXCL12). SDF-1 binding to CXCR4 not only causes the activation of VLA-4 but also cellular polarization leading to directional movement towards soluble SDF-1 gradients. The expression of SDF-1 chemokine is regulated by hypoxia-inducible factor  $1\alpha$ , a transcription factor which responds to low oxygen conditions. Ischemic lesions are for example known to produce SDF-1 causing migration to the injury. Under normal circumstances, the bone marrow is also a relatively hypoxic environment which induces a basal level of SDF-1 expression. *in vitro* experiments testing cell migration in response to SDF-1 eventually led to the use of AMD3100. *in vivo* the initial AMD3100 HSC mobilization study demonstrated peak release after one hour with mobilized progenitor counts and repopulation similar to 4 day G-CSF treatment. When treatments were combined, a synergistic increase was observed in 4 day G-CSF mice treated for 1 hour with AMD3100. It's plausible the synergy

is related to reduced VLA-4 expression by G-CSF but it could also be due to the release of the G-CSF expanded HSC's which are being held in place by an SDF-1 gradient. AMD3100 has been shown to block both the SDF-1 chemotactic gradient and activation of integrins so it is uncertain which effect is more important in mobilization. Regardless, AMD3100 has proven that disruption of the CXCR4 receptor is a viable strategy for HSC mobilization.

VLA-4 mobilization has been researched for almost twenty years. The study by Weissman et. al. mentioned earlier reported that  $\alpha$ 2-6 and  $\beta$ 1 integrins are all expressed by HSC populations (Wagers et al., 2002). The study also reported that an  $\alpha$ 4 antibody can significantly reduce bone marrow homing in mice. A study published a decade earlier in 1993 reported that  $\alpha$ 4 antibody can also cause a 200-fold increase of peripheral HSC's in primates (Papayannopoulou and Nakamoto, 1993). More recently  $\alpha$ 4 mobilization has been reported to cause continuous high levels of circulating HSC's found in multiple sclerosis patients treated with Natalizumab (Jing et al., 2010; Zohren et al., 2008). Unexpectedly, chronic Natalizumab treatment was also reported to cause a  $\approx$ 15 fold HSC population increase in patient bone marrow compared to healthy donors. The cause is uncertain, it could be due to a disruption in growth regulation mediated by  $\alpha$ 4 or possibly a side effect of blocking leukocyte homing associated with inflammation in autoimmune disease. Even more recently a novel VLA-4 targeted molecule, BIO5192 was used to mobilize HSC in mice. Data was collected over a 24 hour collection period, with peak HSC counts after 3-6 hours. BIO5192 is a small molecule inhibitor based on the LDV sequence of fibronectin and known to expose the  $\beta$ 1 LIBS (Leone et al., 2003). It has high selectivity for both active and inactive  $\alpha$ 4 $\beta$ 1, with higher affinity than the natural ligand ( $< 10 \mu\text{M } K_d$ ). Interestingly, BIO5192 was reported to be synergistic with both AMD3100 and G-

CSF separately or combined. This suggests there are separate mechanisms by which each G-CSF, BIO5192 and AMD3100 operate which are all uniquely important in HSC mobilization. Thus, taken together VLA-4 represents a proven target for HSC mobilization and therefore represents a potential therapeutic target for novel *in vivo* phenothiazine treatment.

## 1.5 VLA-4 and Cancer Therapy

In hematological cancers, drug resistance is a major factor which determines patient outcome. Often resistance may go undetected until relapse. This is especially true in minimal residual disease where small numbers of surviving cancer cells can propagate an often resistant malignancy. Two major contributors to drug resistance are location, which affects the effective concentration of drug delivery, and growth-survival signaling, which can overcome a drug's mechanism of action. The bone marrow niche which normally harbors HSC's and facilitates hematopoiesis is thought to be a safe harbor for cancer cells contributing to both aspects of resistance. The niche is isolated from major blood flow and provides necessary ligands to facilitate at least three separate pro-growth pro-survival signaling pathways (McCubrey et al., 2008). Over-time the acceptance and understanding of this niche has broadly expanded treatment design in hematological cancers. Compounds which affect cells in the niche including compounds targeting integrins are now being tested in a multifaceted approach designed to increase efficacy of chemotherapeutic agents.

Integrins are thought to affect three major factors in niche mediated cancer resistance: homing of cancer cells to the bone marrow, retention of cancer cells in the niche, and adhesion mediated pro-survival signaling. The contribution of homing to

malignancy was shown in a study which compared leukemic progression between a wild-type acute lymphoblastic leukemia cell line, Nalm-6, expressing VLA-4 and a mutated version of the cell line lacking  $\beta 1$  (CD49d) expression. All mice injected with Nalm-6 (wt) cells developed cancer involving hind-limb paralysis with an average event free survival of 30 days. In mice injected with Nalm-6 ( $\beta 1^-$ ) only one out of twelve mice developed hind-limb paralysis and six mice were event free after 100 days. Furthermore within the bone marrow VLA-4 integrin also contributes to the retention of these cancer cells. Since, many hematologic cancers tend to be mutated forms of early HSC progenitors they are retained similarly to the normal HSC population. VLA-4 expression has been confirmed on numerous acute lymphoblastic leukemia, acute myeloid leukemia, and multiple myeloma cell lines as well as patient cells (Mudry et al., 2000; Noborio-Hatano et al., 2009; Matsunaga et al., 2003). The natural ligands for this VLA-4 mediated retention are fibronectin in the bone marrow extracellular matrix and VCAM-1 expressed on bone marrow stromal cells. While attachment to these stromal cells serves to isolate the cancer cells from the bloodstream it also contributes to another consequence of VLA-4 adhesion. Binding to ligand also propagates outside-in signaling through a conformational change in the cytoplasmic tails of each VLA-4 subunit. When bound the cytoplasmic tails of VLA-4 untwist allowing the scaffolding of numerous signaling kinases (Figure 2C). In ALL, AML and MM cell lines, binding to fibronectin has been show to activate the pro-survival PI3K/Akt/BcL-2 pathway. Binding to VCAM-1 on marrow stromal cells is known to activate ILK which induces phosphorylation of STAT3, Akt, and ERK, or more simply three separate pro-growth pro-survival pathways. Thus, evidence exists for multiple mechanisms by which VLA-4 can contribute to drug resistance in hematological cancers.



The potential for hematological cancer treatment directed at VLA-4 has been demonstrated both *in vitro* and *in vivo*. *in vitro* studies have reported the ability of VLA-4 directed compounds to overcome the anti-cancer drug resistance derived from binding fibronectin or stromal cells in AML, MM, and ALL (Mudry et al., 2000; Matsunaga et al., 2003; Zeng et al., 2009; Noborio-Hatano et al., 2009). In animal studies at least two different approaches have been tested. One of these studies reported that an antibody to VLA-4 combined with Ara-C increased the overall survival of mice infected with patient AML cells. In multiple myeloma a protease inhibitor, bortezomib, which is thought to down-regulate the  $\beta$ 1 subunit has shown potential in fighting what is a very drug resistant plasma cell cancer (Noborio-Hatano et al., 2009). In fact, bortezomib is now being tested in a number of clinical trials as a potential treatment in MM where to date 10-year survival is almost zero. However, while bortezomib is in clinical trials treatment with other antibodies to VLA-4 and VLA-4 small molecule compounds has not yet reached the broad acceptance needed for clinical trials. A recent review has suggested a number of reasons for the delay (Cox et al., 2010). First, the current clinical drugs natalizumab and efalizumab exhibit a number of side effects including the aforementioned risk of PML. Second, neutralizing antibodies have been shown to develop against antibody-based treatment with natalizumab. Lastly, the small-molecule integrin targeted drugs were developed without extensive knowledge of the role of integrin receptors creating concern over potential agonist properties. Thus, while there is evidence VLA-4 is useful in the treatment of hematological cancer known antibodies and small molecules are not considered ideal clinical candidates.

## 1.6 Experimental Approaches

The 2008 high-throughput screen served to establish the previously unknown relationship between VLA-4 integrin and phenothiazines. The long clinical history of phenothiazines and decades of research on VLA-4 seems to further confirm this relationship. Because of this and because these compounds represent the first known allosteric modulators of VLA-4 many questions were raised concerning the direct nature of this relationship. To investigate these questions a number of *in vitro* and *in vivo* experiments were performed. The first set of experiments asked whether phenothiazines could have the same impact on integrins in the high affinity integrin state as compared to the low affinity integrin state tested in the screen. These studies were carried out using previously established flow cytometry methods to monitor dissociation of ligand in real-time following integrin affinity activation through  $G\alpha_i$  signaling. The next experiments sought to answer whether the phenothiazine compounds were causing ligand dissociation by altering intracellular signaling. Again flow cytometry was used, this time to monitor real-time changes in intracellular calcium concentration as an indicator of signaling. The next investigation was whether phenothiazine could impact cellular homing as seen by aforementioned studies using AMD3100. This was tested *in vitro* using chemotaxis assays to assess cellular migration towards chemokines. The questions then shifted to whether or not phenothiazines could have a measurable impact on a VLA-4 integrin mediated process *in vivo*. Because of the recent success with small molecule VLA-4 inhibitors on HSC mobilization, treatment with phenothiazine was expected to have similar results. Therefore, mobilization was assessed in mice to determine if phenothiazine could increase the circulating HSC population. For this study phenothiazine was injected

one hour prior to blood collection and results were compared to similar treatment with the positive control AMD3100. Finally, because of VLA-4's role in the cancer niche and case studies suggesting phenothiazines played a miraculous role in overcoming drug resistant cancers the question was asked whether phenothiazine could potentiate the effects of anti-cancer drugs by blocking integrins. To test this hypothesis an *in vitro* study was designed using a B-cell acute lymphoblastic leukemia cell line seeded in culture dishes on the VLA-4 ligand VCAM-1 and measuring whether or not phenothiazine increased cell death caused by the anti-cancer drug cytosine arabinoside. Together these experiments should help to determine the impact of phenothiazines as an allosteric modulator of VLA-4 and potentially provide insight towards future therapeutic treatments.

## 2 Methods

### 2.1 Materials

VLA-4 specific ligands LDV and LDV-FITC the VLA-4 specific ligands were synthesized at Commonwealth Biotechnologies (Chigaev et al., 2008). fMLF (N-formyl-L-methionyl-L-leucyl-L-phenylalanine) was purchased from Sigma-Aldrich. Human recombinant CXCL12-SDF-1 $\alpha$  was from R&D Systems. Thioridazine and AMD3100 were purchased from Tocris Biosciences. For the animal study injection solutions were prepared to 200  $\mu$ L for 30 g mice and adjusted to mass for each mouse individually. Thioridazine was prepared from a 10 mM stock in DMSO diluted to yield a 2.5% DMSO solution in sterile saline. AMD3100 was prepared fresh each day,

weighed and diluted in sterile saline. The vehicle solution was 2.5% DMSO in sterile saline. Chemotaxis chambers were polycarbonate transwell inserts designed to create an upper and lower chamber with 8.0  $\mu$ M pores for cell migration manufactured by Corning (Product #3422). Cytosine arabinoside (Ara-C) and sodium bicarbonate were purchased from Sigma-Aldrich. Recombinant human VCAM-1 Fc chimera was from R&D Systems. HBSS media was from Gibco. Fluo-4, AM Calcium dye was purchased from Invitrogen.

## **2.2 Cell lines and Transfectant Constructs**

The human histocytic lymphoma U937 cell line was originally purchased from ATCC. The transfected lines U937FPR (formyl peptide receptor) and U937FPR $\Delta$ ST, a non-desensitizing mutant of FPR, were a gift from Dr. Eric Prossnitz created as described previously (Kew et al., 1997). B78H1 cells subcloned from the murine melanoma B16 cell line were previously transfected with human VCAM-1 as described (Zwartz et al., 2004). The human B cell precursor leukemia NALM-6 cell line was originally purchased from DSMZ.de. All cells were cultured in complete RPMI 1640 supplemented with L-glutamine, penicillin, streptomycin, non-essential amino acids and fetal bovine serum.

## **2.3 Mice**

Male C57Bl6 mice weighing 24-30 g, age 9-13 weeks were purchased from Jackson Laboratories. Mice were acclimated to the animal facility for at least one week, kept on a 12 hr light/dark cycle and fed a standard diet. Experiments were conducted

between 10:00 AM and 12:00 PM (lights on 7:00 AM). Procedures used in this study were conducted by authorized personnel and approved by the Institutional Animal Care and Use Committee. Mice were injected intraperitoneally with vehicle, thioridazine (1.25 mg/kg), or AMD3100 (5 mg/kg) 1-hour prior to blood collection. Prior to and throughout blood collection mice were anaesthetized using isoflurane and monitored for sensitivity. Blood was collected by heart puncture and continued to exsanguination (1-1.4 mL). Blood was collected in a syringe containing 100  $\mu$ L heparin and immediately mixed into a conical tube containing an additional 100  $\mu$ L heparin to prevent clotting. Total blood volume collected was recorded for each mouse.

## 2.4 Small Fluorescent Molecule LDV-FITC Probe

Real-time binding and dissociation experiments using the LDV-FITC probe have previously been described (Chigaev et al., 2008; Zwartz et al., 2004). In short, cells from culture were washed with serum-free RPMI 1640, resuspended at  $1 \times 10^6$  cells/mL in complete media and placed on ice. For each experiment 500  $\mu$ L cells were transferred into a round-bottom sample tube containing a 5x2 mm magnetic stir bar (Big Science, Huntersville NC) and incubated in a 37°C water bath for at least 5 min. After incubation, samples were analyzed on a FACScan flow cytometer using CellQuest software (Becton Dickinson, San Jose, CA) for up to 1024 seconds, while at 37°C and stirred at 300 rpm. Baseline autofluorescence data was collected continuously for 30-120 seconds followed by addition of 8 nM LDV-FITC. The fluorescent signal after addition of the probe was monitored and allowed to reach a stable plateau (80-120 seconds). At this point GPCR ligands, either 100 nM fMLF or 12.5 nM SDF-1 $\alpha$

were added and monitored for up to an additional 200 seconds.

## 2.5 Intracellular Calcium Mobilization

For reference this assay has previously been described (Chigaev et al., 2001). In summary, first U937 $\Delta$ FPR cells were washed and resuspended at  $5 \times 10^6$  cells/mL in HEPES buffer (110 mM NaCl, 10 mM KCl, 1 mM MgCl<sub>2</sub>, 1.5 mM CaCl<sub>2</sub>, 30 mM HEPES, 10 mM glucose, 0.1% HSA, pH 7.4). The cells were then labeled with fluo-4, a green fluorescent calcium dye, at a concentration of 5  $\mu$ g/mL for 30 minutes. After labeling the cells were resuspended at  $1 \times 10^6$  cells/mL in HEPES buffer and stored on ice until used in experiments. For each experiment 500  $\mu$ L of U937 cells were added to round-bottom sample tubes each containing a 5 x 2 mm magnetic stir bar and incubated at 37°C for 3 minutes. After incubation the samples were analyzed on the flow cytometer (FACScan). Samples were stirred continuously at 300 rpm and kept at 37°C. Data was collected as a measure of mean green fluorescence/second. For each sample approximately 30 seconds of baseline signal was collected followed by the addition of 100 nM fMLF. Following activation with fMLF data was collected for 3 additional minutes. In blocking experiments 1.25  $\mu$ L DMSO or 25  $\mu$ M thioridazine was added 90 seconds after the addition of fMLF. In pre-activation experiments DMSO or thioridazine was added at the beginning of the 3 minute incubation period.

## 2.6 Cell Aggregation Assay

For reference this assay has previously been described (Chigaev et al., 2008; Zwartz et al., 2004). In summary, first cell lines U937 $\Delta$ ST, U937 $\Delta$ FPR and B78H1 (har-

vested with 1 mM EDTA) were resuspended in separate 50 mL conical tubes using serum-free RPMI-1640 to remove any serum from the cells. Next, the cells were centrifuged at 1200 rpm for 5 min and aspirated until approximately 25  $\mu$ L of supernatant remained on the cell pellets. At this point the U937 cell lines were stained with red fluorescent PKH26GL dye and the B78H1 cells with green fluorescent PKH67GL dye according to the manufacturer’s instructions (Sigma-Aldrich, 2010). After staining, cells were resuspended in RPMI complete media at concentrations of  $3 \times 10^6$  cells/mL for B78H1 and  $1 \times 10^6$  cells/mL for the U937 cell lines and kept on ice until used in assays. Next, the flow cytometer (FACScan) was prepared for acquisition by analyzing the red and green stained cells individually or mixed together. Discrete populations were set-up for the green FL1 (B78H1 cells), red FL2 (U937 cells), and aggregate fluorescent signals (Figure 4). A gate was then created for the aggregate cell population allowing the acquisition of total events/second. Following instrument set-up, 500  $\mu$ L B78H1 and 500  $\mu$ L U937 cells were added to round-bottom sample tubes each containing a 5 x 2 mm magnetic stir bar. Sample tubes were then placed in a 37°C water bath for at least 5 min. After incubation tubes were attached to the flow cytometer while resting in a water bath maintained at 37°C on top of a magnetic stir plate set to 300 rpm. Aggregation data was then collected for up to 1024 seconds during which time sequential additions of compounds were made to stimulate or inhibit aggregation.

## 2.7 Chemotaxis Assays

From culture, cells were pelleted at low speed in a centrifuge, 7 min at 1000 rpm, decanted, and washed in complete RPMI 1640 media to improve starting viability.

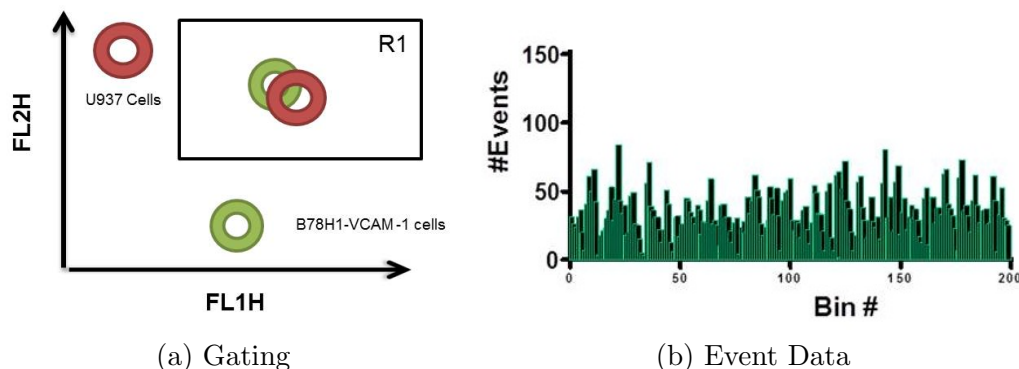
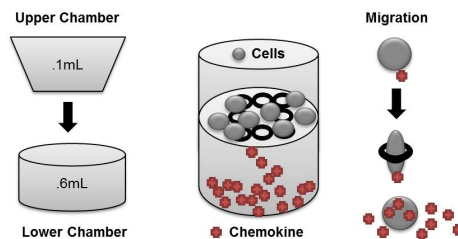


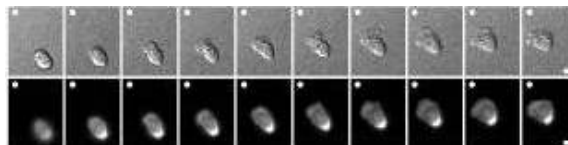
Figure 4: **Aggregation Methods.** (a) Gating schematic for positive selection of U937-B78H1 aggregates; (b) Representative histogram of raw aggregation data displayed as number of events per bin.

Next, cells were counted using a Vi-CELL XR (Beckman-Coulter) automated cell counter and resuspended to approximately  $2 \times 10^6$  cells/mL. Then, 100  $\mu\text{L}$  aliquots of cells were pipetted in succession into transwell inserts. Additional 100  $\mu\text{L}$  aliquots were also collected and re-counted to determine average load. The inserts containing cells were then carefully placed into the wells of 24-well plates (Figure 5). Each plate-well was prepared with 600  $\mu\text{L}$  of room temperature complete RPMI media containing either chemoattractant or PBS. For inhibition assays, thioridazine or AMD3100 were immediately added to the transwell inserts following cell addition. Drug concentration was based on the 100  $\mu\text{L}$ /chamber volume in the inserts. Chemotaxis plates were then incubated for four hours at  $37^\circ\text{C}$  with  $5\% \text{CO}_2$ . After four hours, inserts were removed from all wells and 225  $\mu\text{L}$  sample was collected from each bottom well after uniform mixing. Each 225  $\mu\text{L}$  sample was then diluted into 450  $\mu\text{L}$  PBS and counted using a Vi-Cell XR automated cell counter. Percent migration was determined for each well from the ratio of the total cell/mL count at the end of experiments to the average viable cell/mL count from the beginning of the experiments.





(a) Experiment schematic



(b) Neutrophil chemotaxis (Lokuta et al., 2007)

Figure 5: **Chemotaxis Methods.** (a) Illustrations of the assembled chemotactic apparatus and cellular migration through the transwell pores; (b) Microscopic visualizations of neutrophils undergoing chemotaxis.

## 2.8 Blood Processing and CFU Plating

Blood collected from mice (see above) was processed according to the protocol recommended by StemCell Technologies (StemCell, 2005). Prior to processing, blood was stored at room temperature as collected in 15 mL conical tubes. In brief, blood samples were first lysed in 10-12 mL ammonium chloride lysis buffer (StemCell Technologies) for 10 min on ice. The conical tubes were then centrifuged for 7 min at 1200 rpm. The supernatants were then aspirated to approximately 1 mL and washed with 6 mL PBS (Gibco). The conical tubes were again centrifuged for 7 min at 1200 rpm. The supernatants were aspirated to 0.5 mL and washed with 6 mL IMDM-2%FBS (Sigma). Once again the conical tubes were centrifuged for 7 min at 1200 rpm. The supernatants were aspirated to approximately 50  $\mu\text{L}$  and the remaining pellets were re-suspended in 600  $\mu\text{L}$  IMDM-2%FCS (StemCell Technologies). 56.5  $\mu\text{L}$  from each sample was added to 562.5  $\mu\text{L}$  PBS for nucleated cell enumeration using a Vi-Cell

XR automated cell counter. The measured total cell/mL counts were adjusted for volume collected to determine the white blood cells/mL count. Next, samples were centrifuged for 5 min at 1400 rpm, aspirated and re-suspended in IMDM-2%FCS to achieve  $2 - 3 \times 10^6 \text{ cells/mL}$  based on the total cell/mL count.  $56.5 \mu\text{L}$  aliquots of these new solutions were again diluted in  $562.5 \mu\text{L}$  PBS for counting. These cell counts were the established load counts used to determine CFU/mL.  $300 \mu\text{L}$  of each load sample was then added to a tube containing 3 mL MethoCult (StemCell Technologies) and vortexed for 20 seconds. 1.1 mL of the resulting MethoCult-cell mix was plated in p35 dishes (2 per sample) and incubated at  $37^\circ\text{C}$  with  $5\% \text{CO}_2$ . Colony forming cells were counted 14 days later at 10x magnification.

## **2.9 B-ALL - VCAM-1 cell viability assay**

These experiments were based on previously established methods (Matsunaga et al., 2003; Zeng et al., 2009). In brief, rVCAM-1 was reconstituted in  $100 \mu\text{g/mL}$  PBS and diluted 1:100 in 0.1 M sodium bicarbonate in HBSS.  $100 \mu\text{L}$  of the resulting solution or a VCAM free solution was added to each well of a 12-well plate and incubated at  $4^\circ\text{C}$  for 2 hours. Next, from culture Nalm-6 cells were washed with serum-free RPMI 1640 resuspend at  $0.5 \times 10^6 \text{ cells/mL}$  in complete RPMI 1640 media. For each well 1.5 mL of cells were added followed by DMSO, thioridazine or  $2 \mu\text{M}$  Ara-C. After 24-hours cell viability was assessed using a Vi-CELL XR automated cell counter.

## 2.10 Analysis

Flow cytometry experiments were conducted using a FACScan flow cytometer and Cell Quest software (Becton Dickinson, San Jose, CA). Data from the real-time experiments was converted to mean-FL1H versus time using FCSQuery software (Dr. Bruce Edwards, University of New Mexico). Data from the cell aggregation assay was converted to number of aggregate events/bin using Hyperview software (Dr. Bruce Edwards, University of New Mexico). Chemotaxis, CFU and viability data was processed with Microsoft Excel and GraphPad Prism. CFU counts were averaged across two plates for each mouse and normalized based on the load count to give CFU/ $1 \times 10^6$  cells. That ratio was then normalized to the CFU/mL count based on the total white blood cell count mentioned earlier. Representative images of AMD3100 and thioridazine colony forming cell plates were collected using a dark-field microscope equipped with a CCD camera at 4x magnification. All graphs and kinetic analysis were generated with Prism 4.0 (GraphPad Software).

## 3 Results

Previously, five phenothiazine compounds were selected from the 2008 screen and tested in a flow cytometry assay for the ability of each phenothiazine to induce dissociation of a fluorescent VLA-4 ligand, LDV-FITC, from U937FPR $\Delta$ ST cells (Chigaev et al., 2011). The EC<sub>50</sub> for dissociation of each phenothiazine compound was determined using five serial concentrations. All five compounds had an EC<sub>50</sub> in the range of 3.2 – 13.0 $\mu$ M. Due to this narrow range for integrin modulation the best candidate compound for this research was instead chosen based on highest clinically

safe dose. Background literature indicated the compound thioridazine would allow the highest dose among the five compounds with a published 800 mg/day maximum dose and clinically relevant doses from 2-36  $\mu\text{M}$  plasma concentration (Zhelev et al., 2004; Hartigan-Go et al., 1996; Kemper et al., 1983). Coincidentally, in addition to the highest clinically administered dose, thioridazine also had the lowest  $\text{EC}_{50}$  of 3.2  $\mu\text{M}$  of the five compounds in the aforementioned assay. Hence, thioridazine was chosen as the phenothiazine compound for this research.

### **3.1 Thioridazine strongly dissociates ligands from $\text{G}\alpha_i$ induced high affinity VLA-4 integrins.**

The aforementioned assay established the ability of thioridazine to induce dissociation of the LDV-FITC probe under basal integrin affinity (Chigaev et al., 2011). Here thioridazine was tested against  $\text{G}\alpha_i$  affinity activated VLA-4 integrin adhesion in two cell line constructs, U937FPR and U937FPR $\Delta$ ST. Both cells lines express the  $\text{G}\alpha_i$  coupled formyl peptide receptor. U937FPR cells express a wild-type version of the receptor and U937FPR $\Delta$ ST cells express a non-desensitizing mutant of the receptor which maintains high VLA-4 affinity after stimulation with fMLF chemokine. VLA-4 ligands for these experiments were LDV-FITC and VCAM-1 a natural ligand expressed on B78H1 mouse melanoma cells.

To monitor association and dissociation cells U937FPR (Figure 6a) or U937FPR $\Delta$ ST (Figure 6b) were first incubated with 8 nM LDV-FITC probe. Next, the cells were stimulated; addition of DMSO caused maintenance of basal fluorescence and addition of fMLF at saturating concentration to cause high affinity activation increased

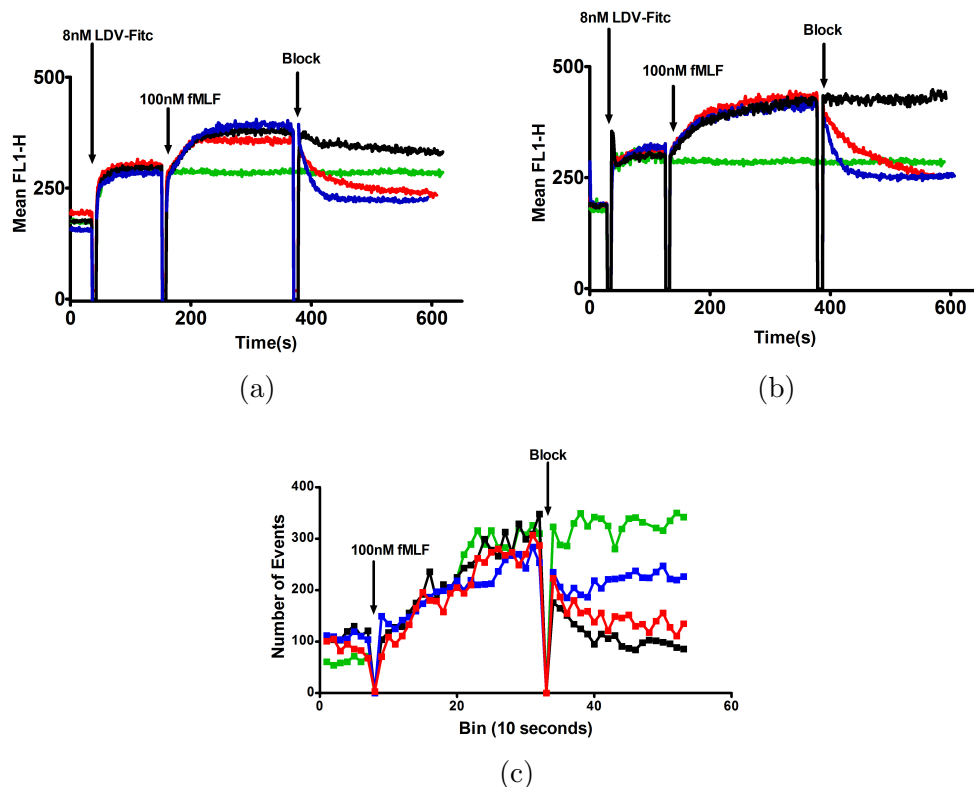


Figure 6: **Real Time Association and Dissociation of LDV-FITC and B78H1 cells in response to thioridazine:** (a) U937FPR or (b) U937FPR $\Delta$ ST association with 8 nM LDV-FITC after stimulation with 100 nM fMLF or DMSO( $\bullet$ ) followed by dissociation after blocking with DMSO( $\bullet$ ), LDV( $\bullet$ ), or 7.5  $\mu$ M thioridazine( $\bullet$ ); and, (c) U937FPR $\Delta$ ST and B78H1VCAM-1 cell aggregation following stimulation with 100 nM fMLF and dissociation after blocking with DMSO( $\bullet$ ), LDV( $\bullet$ ), 10  $\mu$ M thioridazine( $\bullet$ ), or 50  $\mu$ M thioridazine( $\bullet$ ).

binding of LDV-FITC. Following the stimulation with fMLF, peak fluorescence was established. Samples were then treated with DMSO, 1  $\mu$ M non-fluorescent LDV or 7.5  $\mu$ M thioridazine. The addition of thioridazine reduced fluorescent signal to below basal levels and at a faster rate than blocking with LDV. The loss of fluorescence appeared only slightly faster in U937FPR cells compared to the non-desensitizing U937FPR $\Delta$ ST cells.

Next, a similar activation strategy was tested in a method one step closer to the native pathway with VCAM-1 expressing B78H1 cells (Figure 6c). Red stained U937FPR $\Delta$ ST cells were incubated with green stained B78H1 cells causing contact and adhesion of the two cell types. Data were collected as double positive red-green events/10 sec as a measure of VCAM-1 association with VLA-4. Again samples were stimulated with fMLF to activate VLA-4 integrins and to increase association events. After peak association, DMSO, 1  $\mu$ M LDV, 10  $\mu$ M thioridazine, or 50  $\mu$ M thioridazine was added and dissociation was then monitored. Here, the 10  $\mu$ M dose of thioridazine showed decreased dissociation compared to LDV. Increasing the concentration of thioridazine to 50  $\mu$ M caused an incremental effect showing slightly more dissociation than LDV. Thus, together thioridazine was able to disrupt the high affinity association of VLA-4 integrin with both a small molecule ligand and with native ligand.

### **3.2 Thioridazine blocks $G\alpha_i$ chemokine mediated chemotaxis.**

Thioridazine was next assessed for its ability to alter homing *in vitro* using chemotaxis. Chemotaxis utilizes specially designed porous chambers which allow cells to migrate towards chemokine gradients in a bottom chamber. Successful migration requires cell polarization and motility to reach and pass through the small pores. To examine chemotaxis first wild type U937FPR and mutant U937FPR $\Delta$ ST migration were compared using the two separate chemokines fMLF and SDF-1 $\alpha$  to induce chemotaxis (Figure 7a). The U937 cells were added to the upper wells of chemotaxis chambers with lower chambers containing media and PBS, SDF-1 $\alpha$  or fMLF. After four hours, cells in lower chambers were counted and compared to the number

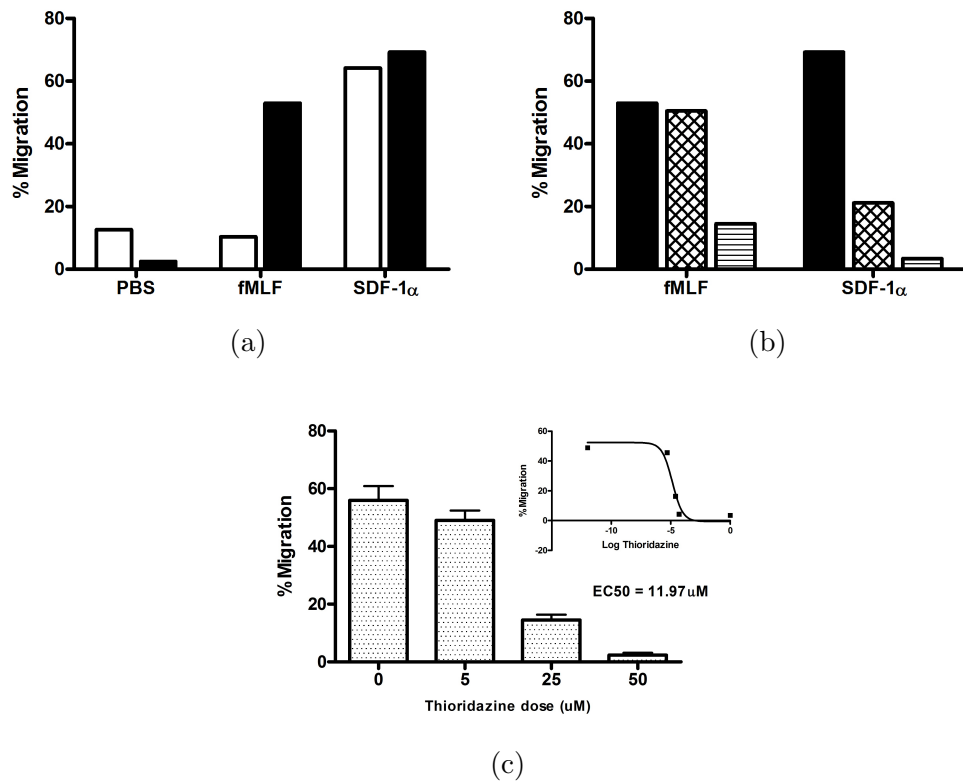


Figure 7: **Effects of thioridazine on U937 cell migration:** (a) U937FPR $\Delta$ ST(□) and U937FPR(■) chemotaxis to PBS, 100 nM fMLF, or 12.5 nM SDF-1 $\alpha$ ; (b) U937FPR cell chemotaxis to 100 nM fMLF or 12.5 nM SDF-1 $\alpha$  inhibited with PBS(■), 65  $\mu$ M AMD(⊠), or 25  $\mu$ M thioridazine(⊞); and, (c) U937FPR chemotaxis to 100 nM fMLF with increasing concentrations of thioridazine.

of cells loaded into upper chambers to calculate percent migration. Results indicated U937FPR cells could migrate in response to both fMLF and SDF-1 $\alpha$  whereas, U937FPR $\Delta$ ST cells only migrated toward SDF-1 $\alpha$ . Previous research has demonstrated the ST mutants do migrate towards fMLF but an inability to desensitize prevents release into the lower chamber (Zigmond and Hirsch, 1973). Thus this experiment may reflect the importance of integrin adhesion in chemotaxis.

Next, thioridazine was used in the assay to determine if it could alter SDF-

1 $\alpha$  or fMLF mediated chemotaxis (Figure 7B). U937FPR cells were placed in upper chambers containing DMSO, 65  $\mu$ M AMD3100 or 25  $\mu$ M thioridazine with lower chambers containing media and SDF-1 $\alpha$  or fMLF. The assays were again run for four hours and percent migration was calculated for each of the chambers. SDF-1 $\alpha$  chemotaxis was inhibited by both AMD3100 and thioridazine while fMLF chemotaxis was inhibited only with thioridazine. Multiple concentrations of thioridazine were then tested to attain the EC<sub>50</sub> dose for inhibition of fMLF mediated chemotaxis (Figure 7C). Statistical results indicated a 12  $\mu$ M EC<sub>50</sub> for inhibition of migration. Thus thioridazine was able to inhibit chemotaxis to multiple G $\alpha_i$  coupled chemokines at  $\approx 10^{-5}$  molar concentrations.

### 3.3 Thioridazine alters intracellular calcium signaling.

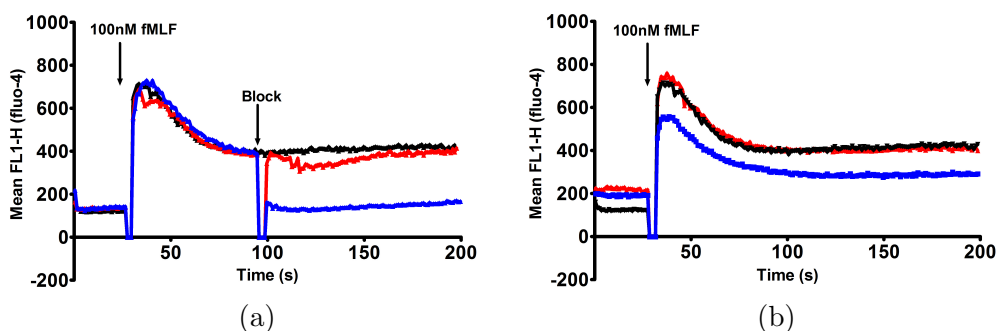


Figure 8: **Real time measurements of intracellular calcium mobilization response to thioridazine:** (a) Calcium mobilization in U937FPR following stimulation with 100 nM fMLF only ( $\bullet$ ) or fMLF stimulation followed by either DMSO ( $\circ$ ) or 25  $\mu$ M thioridazine ( $\circ$ ); and, (b) calcium mobilization with 100 nM fMLF only ( $\bullet$ ) or after pre-incubation with DMSO ( $\circ$ ) or 25  $\mu$ M thioridazine ( $\circ$ ) for 3 minutes.

Next, to determine the impact of thioridazine on intracellular signaling flow cytometry experiments were conducted to monitor intracellular calcium (intracellular



Ca<sup>2+</sup>) mobilization. Cell stimulation with fMLF is known to cause a spike intracellular Ca<sup>2+</sup> which correlates to VLA-4 extension and increased affinity (Chigaev et al., 2007). Here thioridazine was added prior to or following fMLF stimulation to determine the impact on intracellular Ca<sup>2+</sup>. U937FPR cells were labeled with the green fluorescent intracellular Ca<sup>2+</sup> probe fluo-4. The fluo-4 probe displays increased fluorescent emission upon binding calcium. This change in fluo-4 signaling was monitored in real-time with flow cytometry. Calcium was mobilized with excess fMLF which caused a rapid 5-fold spike in fluorescence signal. The addition of thioridazine resulted in two different observations. In experiments where thioridazine was added following stimulation (Figure 8a), the fluorescent signal was immediately abrogated to the baseline level. In experiments where thioridazine was added 3 minutes prior to stimulation (Figure 8b), fMLF mediated signaling was only partially reduced. Together, the results indicate that thioridazine does antagonize the signaling related to G $\alpha_i$  integrin activation.

### **3.4 Thioridazine mobilizes hematopoietic progenitor populations *in vivo*.**

Previous studies suggested HSC mobilization could serve as model to determine if *in vitro* integrin inhibition could translate to *in vivo* conditions. Here thioridazine was compared side by side with AMD3100 mobilization as a positive control in murine hematopoietic progenitor cell mobilization. Mice were administered doses of vehicle, AMD3100, or thioridazine. Blood was collected for assessment one hour post injection. First nucleated cell counts were gathered after lysing the red blood cells

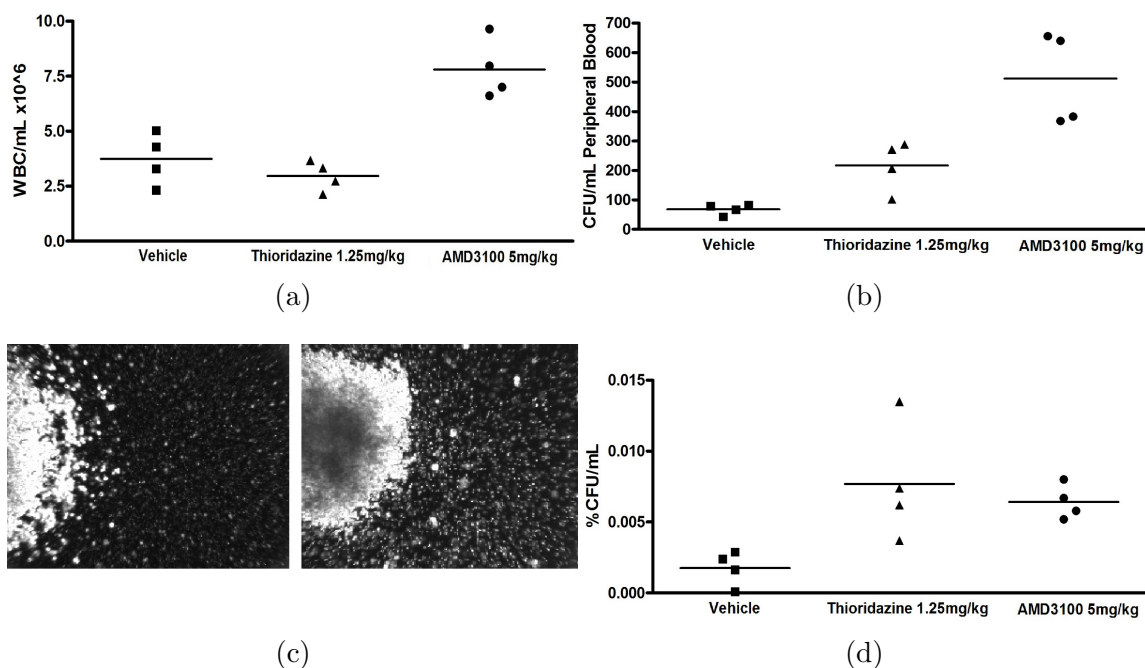


Figure 9: **Hematopoietic progenitor cell mobilization in response to thioridazine or AMD3100:** (a) Displays total white blood cell/mL counts in response to a 1-hour dose of thioridazine or AMD3100 in C57BL6 mice; (b) Release of colony forming cells in response to a one hour dose of thioridazine or AMD3100; (c) Representative dark-field images of CFU colonies displaying the background white blood cell levels in thioridazine treated mice(left) compared to AMD3100 treated mice (right); and, (d) Colony forming cell counts displayed relative to white cell counts displayed as a percentage of CFU/mL.

(Figure 9a). AMD3100 showed significantly increased white/nucleated blood cells compared to both vehicle and thioridazine. Next, cells were counted and plated for colony forming unit(CFU) assays. CFU plates were processed following 14 days of incubation by enumerating individual colonies. These counts were quantified based on plate load and total white blood cells per mL to give total CFU/mL counts (Figure 9b). Results indicated that thioridazine caused increased colonies, three fold higher, relative to vehicle but not as markedly as AMD3100, which showed a six fold increase in colonies over vehicle.

The discrepancy, however, between AMD3100 mobilization which showed much higher WBC counts relative to thioridazine and vehicle indicated perhaps that AMD3100 was having a broader effect on leukocyte populations. Representative photographs of CFU colonies were taken to demonstrate the large difference in the peripheral WBC cells between thioridazine treated mice and AMD3100 treated mice (Figure 9c). Data was then reassessed to normalize the CFU counts/WBC counts (Figure 9d). The normalized counts indicated that the relative percentage of progenitor cells/mL of blood were similar between AMD3100 and thioridazine. While total mobilization with AMD3100 is still higher thioridazine causes a more enriched progenitor and stem cell population. Thus together thioridazine does mobilize colony forming cells but with a lack of the broad leukocyte mobilization seen with AMD3100.

### **3.5 Thioridazine kills B-ALL cells independent of VCAM-1 and the anti-cancer drug Ara-C.**

To determine the impact of allosteric VLA-4 inhibition in cancer treatment cell viability was assessed in B-ALL Nalm-6 cells seeded on rVCAM following overnight treatment with thioridazine (Figure 10). Previous studies have demonstrated attachment to fibronectin, VCAM-1 or stromal cells causes anti-cancer drug resistance in AML, ALL and MM cell lines. Here, Nalm-6 cells were pre-incubated in cell culture plates seeded with recombinant VCAM-1. Four hours later, DMSO, Ara-C (cytosine-arabinoside) or varying concentrations of thioridazine were added and the plates were incubated overnight. Following the 24 hour incubation, cell viability was measured for each sample. Incubation with Ara-C stopped proliferation and lowered

viability slightly. Thioridazine displayed a dose dependent toxicity starting at the 10  $\mu\text{M}$  dose. This matched a previous study which tested thioridazine toxicity in multiple leukemic cell lines Zhelev et al. (2004). Seeding with rVCAM-1 had no effect on drug resistance even when testing other methods to elicit VCAM-1 rescue. The other tests included altering thioridazine exposure time, pre-incubation times, VCAM-1 concentration and the removal of unattached cells (unpublished). Thus these results indicate that thioridazine is toxic to B-ALL cells over 24 hour periods starting at low  $10^{-5}$  molar concentrations.

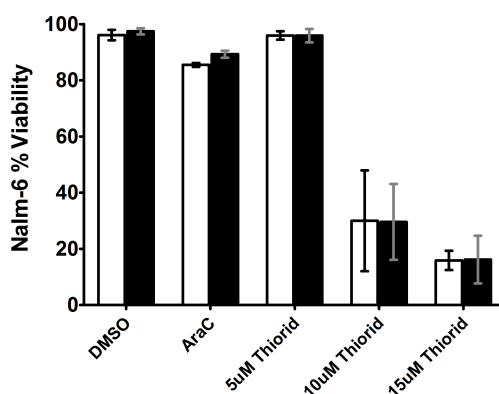


Figure 10: **Nalm-6 cell viability in response to treatment with thioridazine or Ara-C:** Displays the viability of Nalm-6 cells (B-ALL cell line) following 24 hour treatment using the anti-cancer drug Ara-C or various concentrations of thioridazine. The drugs were tested for integrin-adhesion induced resistance with(■) rVCAM-1 seeding or without(□).

## 4 Discussion

### 4.1 Effects of phenothiazines on VLA-4 affinity and cell adhesion.

The properties and kinetics of VLA-4 activation have been well characterized by previous studies using undifferentiated U937 cells in a model system (Chigaev et al., 2007). More recently a HUTS-21 based primary drug screen and pilot study established that thioridazine and other phenothiazines can reduce HUTS-21 binding, LDV-FITC binding, and VCAM-1 dependent aggregation using the same model system (Chigaev et al., 2011). In this study, thioridazine was further tested following  $G\alpha_i$  stimulation which is known to cause both increased ligand affinity and integrin extension through intracellular signaling pathways (Chigaev et al., 2009). The stimulation of these two integrin properties has multiple implications *in vivo* including cellular tethering, firm adhesion, homing, and localization. The results here demonstrated that thioridazine still reduces both LDV-FITC binding and VCAM-1 dependent aggregation following stimulation with a  $G\alpha_i$  signal inducing chemokine (Figure 6).

Furthermore, these experiments found the loss of LDV-FITC binding signal using thioridazine to be accelerated relative to the loss of fluorescence using unlabeled LDV (Figure 6a, 6b). A similar accelerated off rate has been shown previously in a study using the compound U-73122, known as a phospholipase C inhibitor (Chigaev et al., 2007). The comparable off rate to U-73122 may be a clue as to the mechanism of action and target by which thioridazine modulates VLA-4. Interestingly, U-73122 has also been shown to inhibit neutrophil and platelet aggregation (Bleasdale et al.,

1990). Together these results indicate allosteric antagonists such as thioridazine can, in this  $G\alpha_i$  stimulation model cause accelerated inhibition of VLA-4 related adhesion relative to direct VLA-4 compounds such as LDV.

Of note, an increased concentration of thioridazine was required to disaggregate B78H1-VCAM-1 from the U937 cells (Figure 6c). Initially, at the 10  $\mu\text{M}$  concentration thioridazine did reduce aggregation but less so than LDV. Once thioridazine concentration was increased to 50  $\mu\text{M}$  the initial rate of disaggregation was similar to that of the LDV-FITC off rate. It is important to take this finding into consideration since this model system is considered one step closer to the native pathway of integrin cellular adhesion. The lowered efficacy seen here may suggest a potential limitation to future studies concerning thioridazine's therapeutic efficacy. One reason for the the decreased efficacy could be due to the multivalent interactions that occur with aggregation relative to the single receptor-ligand interactions that occur in the LDV-FITC experiments. Previous experiments have found that U937 cells express approximately 55,000 VLA-4 receptors, while VCAM-1 transfected B78H1 cells averaged approximately 500,000 surface ligands (Zwartz et al., 2004). Since this model is only a measure of complete disaggregation, the additional requirement to interrupt multiple receptors for disaggregation is therefore one possible reason for thioridazine's reduced efficacy. The decreased efficacy could have also been caused by non-specific binding between thioridazine and B78H1 cells. The B78H1 cells were added at a three to one ratio with the U937 cells, which also increased total cell concentration by three fold per sample. If then, total thioridazine binding was similar across the two cell types, the concentration difference alone could have accounted for the decrease in efficacy.

## 4.2 Effects of phenothiazines on intracellular signaling.

Intracellular  $\text{Ca}^{2+}$  signaling was assessed to investigate the mechanism by which thioridazine was modulating VLA-4. The results showed that thioridazine significantly reduced intracellular  $\text{Ca}^{2+}$  levels in  $G\alpha_i$  stimulated U937 cells (Figure 8a). Following the addition of thioridazine the loss of intracellular  $\text{Ca}^{2+}$  fluorescent signal was seen as instantaneous relative to the precision of the experiment. The loss of signal appears to correlate with the rapid off rates observed in the LDV-FITC and B78H1 experiments (Figure 6a, 6b). Intracellular  $\text{Ca}^{2+}$  flux is known to be a key signal in the integrin activation pathway caused by  $G\alpha_i$  stimulation. It is known to contribute to the molecular unbending of VLA-4 and subsequently mediate both cellular arrest and firm adhesion.

The rapid loss of intracellular  $\text{Ca}^{2+}$  signal is similar to results from two previous studies, both using the aforementioned phospholipase C inhibitor, U-73122 (Chigaev et al., 2007; Hyduk et al., 2007). The continued similarities between this phospholipase C inhibitor and thioridazine suggests signaling disruption as a possible pathway through which thioridazine disrupts  $G\alpha_i$  activated VLA-4 integrin. However, in the initial pilot study thioridazine altered resting state VLA-4 integrin on unstimulated U937 cells, but as shown in Figure 8, prior to  $G\alpha_i$  stimulation U937 cells have only limited basal levels of free intracellular  $\text{Ca}^{2+}$  (Chigaev et al., 2011). Together, these findings indicate there are potentially two mechanisms by which thioridazine can modulate VLA-4. One mechanism, shown here, which disrupts signaling related to activated VLA-4 integrin, and the second mechanism, which allosterically inhibits VLA-4 in the resting state. Considering that phenothiazines have been tied to numerous receptors and widespread activities against various diseases this possibility of

multiple activities related to VLA-4 is within reason (Sudeshna and Parimal, 2010; Ohlow and Moosmann, 2011). Still, the counter argument exists that the mechanism affecting the resting state also affects the activated state. As of now, U-73122 has not been tested under resting conditions. It is therefore possible that U-73122 also acts as an allosteric inhibitor in the resting state. To make the distinction the first study should be to investigate known phospholipase C inhibitors, such as U-73122, to determine if they can also affect resting VLA-4 similarly to thioridazine. Future studies should also aim to uncover the activities related to thioridazine's resting state allosteric modulation. Temperature, has already been shown to have no effect on disruption, further studies could examine thioridazine's effects on mechanical conformation and whole cell membrane potential. (Chigaev et al., 2011).

### **4.3 Effects of phenothiazines on cellular motility.**

The  $G\alpha_i$  chemokines which upregulate VLA-4 affinity and extension also create diffuse gradients *in vivo* which induce cellular polarity, and cause cellular localization. As such, the next set of experiments assessed the impact of thioridazine on *in vitro* chemokine induced cellular motility. Since AMD3100 was planned as the positive control for the *in vivo* HSC mobilization experiments, these chemotaxis assays were performed comparing thioridazine's impact on cell motility with AMD3100. Here, thioridazine inhibited mobilization to two separate  $G\alpha_i$  chemokines while AMD3100 only inhibited SDF-1 $\alpha$  mobilization (Figure 7b). Taken together with the flow cytometry experiments these results further suggest thioridazine inhibits  $G\alpha_i$  mediated chemotaxis through the broad inhibition of adhesion signaling while AMD3100 only impacts signaling through its known target, the CXCR-4 receptor. For complete-



ness a comparison study was conducted between U937FPR $\Delta$ ST and U937FPR cells which indicated the importance of steady state activation of the  $G\alpha_i$  receptors during motility (Figure 7a). As has been described previously the U937FPR $\Delta$ ST cells appeared unable to detach from the transwell inserts due to the constitutive activation mediated by the ST variant of the FPR receptor.

#### **4.4 Potential for blocking VLA-4 with phenothiazines in cancer treatment.**

Previous studies had found that both AMD3100 and VLA-4 antibodies were capable of potentiating Ara-C cytotoxicity against attached leukemic cell lines (Zeng et al., 2009; Matsunaga et al., 2003). In this study thioridazine alone markedly decreased cell viability of a B-cell acute lymphoblastic leukemia cell line over a 24-hour period in both the presence and absence of VCAM-1 (Figure 10). Compared to the initial prediction that thioridazine would serve to potentiate Ara-C toxicity these results were unexpected. Both the cytotoxicity of thioridazine and the lack of VCAM-1 mediated resistance raised concern. A re-examination of previous studies did however indicate that drug resistance can vary depending on the particular cancer cell lines, and the VLA-4 substrate ligands, among which are fibronectin, stromal cells, and VCAM-1 (Mudry et al., 2000). It is therefore possible that the rVCAM-1 used here was not the correct ligand to mediate resistance. Thus, while there is previous evidence for phenothiazine use against hematological malignancy, in this study VLA-4 inhibition was not confirmed as the mechanism (Jones, 1996). A follow-up study using alternate cell lines and the correct VLA-4 ligands would hopefully address the

limited result seen here.

The other concern raised from these experiments was cytotoxicity. This, however, was also addressed in a previous study which demonstrated that thioridazine also shows a cytotoxic effect in seven other leukemic cell lines (Zhelev et al., 2004). Initially that would be of considerable concern, however, the researchers also tested thioridazine against normal lymphocytes and found no decrease in viability and actually saw an increase in ATP production at higher concentrations of drug. This information along with the knowledge that thioridazine and other phenothiazines have been administered chronically in patients at higher peak blood concentration than the toxicity level found here suggests cytotoxicity may only be relevant to certain cell populations. With respect to the current study, the rapid changes seen in integrin modulation during the flow cytometry experiments were likely unaffected by cytotoxicity since each experiment only had minutes of exposure. There is however, the potential that cytotoxicity played a role in the 4-hour chemotaxis experiments.

#### **4.5 Effects of phenothiazines on HSC mobilization.**

Hematopoietic stem cell mobilization was chosen as the *in vivo* method to test phenothiazine allosteric-modulation of VLA-4 integrin due to the availability of positive controls, previous studies showing VLA-4 expression on the HSC populations, and studies already using direct VLA-4 ligand compounds for mobilization. AMD3100 was used as the positive control due to its well established dose of 5mg/kg and 1-hour time course for peak mobilization. Thioridazine's ability to rapidly dissociate ligand during *in vitro* work suggested a similar time course to AMD3100 would work. Results from these experiments demonstrated that thioridazine could signifi-

cantly increase CFU counts relative to vehicle controls, however, total mobilization was significantly lower than AMD3100 mobilization (Figure 9b–9d). Interestingly, white cell counts obtained prior to CFU plating indicate AMD3100 was not only mobilizing progenitors but also large quantities of additional leukocytes (Figure 9a). Thioridazine meanwhile showed no difference in white cell count compared to vehicle. The varied effectiveness further indicates different mechanisms of action for thioridazine and AMD3100 mobilization. The data suggests that thioridazine has a reduced impact on total leukocyte populations and more focally impacts stem cell populations relative to AMD3100. One reason for this may be due to the increased VLA-4 expression shown previously on early progenitor cells compared to mature leukocytes (Wagers et al., 2002).

Peak HSC mobilization by thioridazine was lower than that of AMD3100 and BIO5192, which was used in a previous study at Washington University in St. Louis (Ramirez et al., 2009). This lower outcome was unexpected following the relatively increased effects of thioridazine in the *in vitro* experiments. A reassessment of the hypothesis relative to these results suggests a number of reasons for reduced mobilization. First, thioridazine blocked chemotaxis to multiple  $G\alpha_i$  chemokines compared to AMD3100 which only inhibited SDF-1 $\alpha$  chemotaxis. Thioridazine also caused a rapid reduction in intracellular  $Ca^{2+}$  signaling. Initially both of these results were thought to be positive indicators of thioridazine’s potential *in vivo*, however, it now seems that while thioridazine can rapidly inhibit integrins, the broad reduction in  $G\alpha_i$  signaling may be effectively causing cellular paralysis. In other words, it’s likely that the stem cells detached but without  $G\alpha_i$  signaling along with the reduced blood flow in the bone marrow niche it may have been unlikely for the cells to enter the blood stream in high numbers. The second explanation for reduced mobilization

could be related to the untested variables dose and time. The optimal time course for AMD3100 mobilization is one hour, the optimal time course for BIOM5192 mobilization was thirty minutes to one hour, and previous work with antibodies has shown peak mobilization at 24 hours (Ramirez et al., 2009; Broxmeyer et al., 2005; Papayannopoulou and Nakamoto, 1993). Here, we only tested one hour time courses for both thioridazine and AMD3100. An important future experiment would be to try other dosing strategies and different time courses to optimize the thioridazine mobilization protocol. Finally, only five phenothiazines were tested in the primary screen and pilot study from which thioridazine was chosen while over sixty phenothiazine compounds have been used clinically. Since thioridazine's efficacy was also reduced going from the LDV-FITC model to the VCAM-1 aggregation model it is suggestive that an alternate phenothiazine compound may produce a larger increase in stem cell release.

Overall though, a positive result was achieved which is important for two reasons. The first reason dates back to the observational study in the 1960's which reported increased atypical lymphocytes in phenothiazine treated patients (Fieve et al., 1966). The clinicians who published the study mentioned in their discussion that the atypical lymphocytes may have been stem cells that had escaped into the blood. Their data are comparable to the *in vivo* mice data seen here. Their data indicates a two-fold increase in the percentage of circulating atypical cells relative to controls while this data shows a two to three fold increase in colony forming units. Thus, this study now answers the question as to why phenothiazine treatment increased peripheral atypical lymphocyte numbers and confirms that the cells indeed represent stem cells or other early hematopoietic progenitor cells. These findings also provide a reason why various blood dyscrasias can occur from phenothiazine treat-

ment (Stübner et al., 2004). Combined these studies further affirm this relationship between phenothiazines and cellular adhesion. The second important point is the affirmation of allosteric VLA-4 modulation as a potential target *in vivo*. While the final result with phenothiazine may be due to a the broad inhibition of adhesion signaling each experiment in the study was carried out based on previous work using direct VLA-4 antibodies and small molecule modulators.

## 4.6 Conclusions

The progression of integrin-directed compounds into clinical trials has been slow with current pharmacological agonists, many of which were developed when integrins were not fully understood (Cox et al., 2010). This study has built on a ten year effort into the understanding of VLA-4 structure and regulation which yielded the discovery of the first known allosteric VLA-4 antagonists potentially representing much better clinical compounds in a number of therapies. The aims were to further characterize the relationship of these novel compounds to VLA-4 modulation and translate the activity to an *in vivo* animal model of hematopoietic stem cell mobilization. Experiments were conducted in a stepwise fashion beginning with well-established *in vitro* models addressing adhesion relevant to the current understanding of HSC mobilization. The results here have established potential for VLA-4 inhibition with phenothiazines against a number of adhesion events including rolling, tethering, firm adhesion, resting adhesion, homing, extravasion, inside-out and outside-in signaling. Due to the long clinical history of phenothiazines these results have also yielded answers to multiple questions concerning side effects of treatment with phenothiazines dating back over forty years. Characterization of this novel relationship also suggests

why both VLA-4 and phenothiazines are being studied and patented independently in a number of the same autoimmune disorders. Further, the similar effects on both intracellular calcium signaling and LDV-FITC fluorescence suggests a link between thioridazine and U-73122 the phospholipase C inhibitor. This finding could potentially represent a previously unknown mechanism and target for phenothiazine action, a topic that is still very controversial in over a century of phenothiazine clinical use (Sudeshna and Parimal, 2010; Ohlow and Moosmann, 2011). The broad inhibition of resting VLA-4, activated VLA-4, intracellular  $\text{Ca}^{2+}$ , and cellular motility indicate why phenothiazines may have such diverse clinical and therapeutic efficacy. Ultimately, thioridazine was successfully translated to an *in vivo* model of HSC mobilization in mice confirming the potential for allosteric VLA-4 modulation *in vivo*. Numerous future experiments will be needed to optimize mobilization, determine effects on other integrins, further address the phospholipase C relationship and establish models in other therapies including cancer.

## 5 References

- R. Alon and K. Ley. Cells on the run: shear-regulated integrin activation in leukocyte rolling and arrest on endothelial cells. Current opinion in cell biology, 2008. URL <http://linkinghub.elsevier.com/retrieve/pii/S0955067408000744>.
- R. Alon and Z. Shulman. Chemokine triggered integrin activation and actin remodeling events guiding lymphocyte migration across vascular barriers. Experimental cell research, 317(5):632–41, Mar. 2011. ISSN 1090-2422. doi: 10.1016/j.yexcr.2010.12.007. URL <http://www.ncbi.nlm.nih.gov/pubmed/21376176>.
- M. A. Arnaout, B. Mahalingam, and J.-P. Xiong. Integrin structure, allostery, and bidirectional signaling. Annual review of cell and developmental biology, 21:381–410, Jan. 2005. ISSN 1081-0706. doi: 10.1146/annurev.cellbio.21.090704.151217. URL <http://www.ncbi.nlm.nih.gov/pubmed/16212500>.
- M. J. Billard and B. W. McIntyre. Access : CD45RA T-cell activation without proliferation by a partial agonist monoclonal antibody to  $\alpha$ 1 integrin : Immunology and Cell Biology. Immunology and cell biology, 86(4):381–4, Jan. 2008. ISSN 0818-9641. doi: 10.1038/sj.icb.7100165. URL <http://dx.doi.org/10.1038/sj.icb.7100165>.

- J. E. Bleasdale, N. R. Thakur, R. S. Gremban, G. L. Bundy, F. A. Fitzpatrick, R. J. Smith, and S. Bunting. Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. The Journal of pharmacology and experimental therapeutics, 255(2):756–68, Nov. 1990. ISSN 0022-3565. URL <http://www.ncbi.nlm.nih.gov/pubmed/2147038>.
- H. E. Broxmeyer, C. M. Orschell, D. W. Clapp, G. Hancoc, S. Cooper, P. A. Plett, W. C. Liles, X. Li, B. Graham-Evans, T. B. Campbell, G. Calandra, G. Bridger, D. C. Dale, and E. F. Srouf. Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. The Journal of experimental medicine, 201(8):1307–18, Apr. 2005. ISSN 0022-1007. doi: 10.1084/jem.20041385. URL <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2213145&tool=pmcentrez&rendertype=abstract>.
- J. A. Burger and T. J. Kipps. CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. Blood, 107(5):1761–7, Mar. 2006. ISSN 0006-4971. doi: 10.1182/blood-2005-08-3182. URL <http://www.ncbi.nlm.nih.gov/pubmed/16269611>.
- G. A. Challen, N. Boles, K. K.-Y. Lin, and M. A. Goodell. Mouse hematopoietic stem cell identification and analysis. Cytometry. Part A : The journal of the International Society for Analytical Cytology, 75(1):14–24, Jan. 2009. ISSN 1552-4930. doi: 10.1002/cyto.a.20674. URL <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2640229&tool=pmcentrez&rendertype=abstract>.
- A. Chigaev, A. M. Blenc, J. V. Braaten, N. Kumaraswamy, C. L. Kepley, R. P. Andrews, J. M. Oliver, B. S. Edwards, E. R. Prossnitz, R. S. Larson, and



- L. A. Sklar. Real time analysis of the affinity regulation of alpha 4-integrin. The physiologically activated receptor is intermediate in affinity between resting and Mn(2+) or antibody activation. The Journal of biological chemistry, 276 (52):48670–8, Dec. 2001. ISSN 0021-9258. doi: 10.1074/jbc.M103194200. URL <http://www.ncbi.nlm.nih.gov/pubmed/11641394>.
- A. Chigaev, L. A. Sklar, A. Waller, T. Buranda, and G. Zwartz. Regulation of Cell Adhesion by Affinity and Conformational Unbending of alpha4beta1 Integrin. The Journal of Immunology, 2007. URL <http://www.jimmunol.org/cgi/content/abstract/178/11/6828>.
- A. Chigaev, A. Waller, O. Amit, and L. A. Sklar. Galphas-coupled receptor signaling actively down-regulates alpha4beta1-integrin affinity: a possible mechanism for cell de-adhesion. BMC immunology, 9:26, Jan. 2008. ISSN 1471-2172. doi: 10.1186/1471-2172-9-26. URL <http://www.ncbi.nlm.nih.gov/pubmed/18534032>.
- A. Chigaev, A. Waller, O. Amit, L. Halip, C. G. Bologna, and L. A. Sklar. Real-time analysis of conformation-sensitive antibody binding provides new insights into integrin conformational regulation. The Journal of biological chemistry, 284(21):14337–46, May 2009. ISSN 0021-9258. doi: 10.1074/jbc.M901178200. URL <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2682882&tool=pmcentrez&rendertype=abstract>.
- A. Chigaev, Y. Wu, D. B. Williams, Y. Smagley, and L. a. Sklar. Discovery of very late antigen-4 (VLA-4, alpha4beta1 integrin) allosteric antagonists. The Journal of biological chemistry, 286(7):5455–63, Feb. 2011. ISSN 1083-

- 351X. doi: 10.1074/jbc.M110.162636. URL <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3037658&tool=pmcentrez&rendertype=abstract>.
- D. Cox, M. Brennan, and N. Moran. Integrins as therapeutic targets: lessons and opportunities. Nature Reviews. Drug Discovery, 9(10):804–20, Oct. 2010. ISSN 1474-1784. doi: 10.1038/nrd3266. URL <http://www.ncbi.nlm.nih.gov/pubmed/20885411>.
- S. Darkin, J. McQuillan, and R. K. Ralph. Chlorpromazine: a potential anticancer agent? Biochemical and biophysical research communications, 125(1):184–91, Nov. 1984. ISSN 0006-291X. URL <http://www.ncbi.nlm.nih.gov/pubmed/6439202>.
- R. R. Fieve, B. Blumenthal, and B. Little. The relationship of atypical lymphocytes, phenothiazines, and schizophrenia. Archives of general psychiatry, 15(5):529–34, Nov. 1966. ISSN 0003-990X. URL <http://www.ncbi.nlm.nih.gov/pubmed/5926600>.
- K. Hartigan-Go, D. N. Bateman, G. Nyberg, E. Mårtensson, and S. H. Thomas. Concentration-related pharmacodynamic effects of thioridazine and its metabolites in humans. Clinical pharmacology and therapeutics, 60(5):543–53, Nov. 1996. ISSN 0009-9236. doi: 10.1016/S0009-9236(96)90150-2. URL <http://www.ncbi.nlm.nih.gov/pubmed/8941027>.
- S. J. Hyduk, J. R. Chan, S. T. Duffy, M. Chen, M. D. Peterson, T. K. Waddell, G. C. Digby, K. Szaszi, A. Kapus, and M. I. Cybulsky. Phospholipase C, calcium, and calmodulin are critical for alpha4beta1 integrin affinity up-regulation and monocyte arrest triggered by chemoattractants. Blood, 109(1):176–84, Jan. 2007.

ISSN 0006-4971. doi: 10.1182/blood-2006-01-029199. URL <http://www.ncbi.nlm.nih.gov/pubmed/16960156>.

Y. Imai, M. Shimaoka, and M. Kurokawa. Essential roles of VLA-4 in the hematopoietic system. International journal of hematology, 91(4):569–75, May 2010. ISSN 1865-3774. doi: 10.1007/s12185-010-0555-3. URL <http://www.ncbi.nlm.nih.gov/pubmed/20352381>.

D. Y. Jackson. alpha 4 integrin antagonists. Current pharmaceutical design, 8(14):1229–53, Jan. 2002. ISSN 1381-6128. URL <http://www.ncbi.nlm.nih.gov/pubmed/12052218>.

D. Jing, U. Oelschlaegel, R. Ordemann, K. Hölig, G. Ehninger, H. Reichmann, T. Ziemssen, and M. Bornhäuser. CD49d blockade by natalizumab in patients with multiple sclerosis affects steady-state hematopoiesis and mobilizes progenitors with a distinct phenotype and function. Bone marrow transplantation, 45(10):1489–96, Oct. 2010. ISSN 1476-5365. doi: 10.1038/bmt.2009.381. URL <http://www.ncbi.nlm.nih.gov/pubmed/20098455>.

K. P. Johnson. Natalizumab (Tysabri) treatment for relapsing multiple sclerosis. The neurologist, 13(4):182–7, July 2007. ISSN 1074-7931. doi: 10.1097/01.nrl.0000263760.53418.5b. URL <http://www.ncbi.nlm.nih.gov/pubmed/17622909>.

G. R. Jones. Successful cancer therapy with promethazine: The rationale. Medical hypotheses, 46(1):25–9, Jan. 1996. ISSN 0306-9877. URL <http://www.ncbi.nlm.nih.gov/pubmed/8746124>.

A. J. Kemper, R. Dunlap, and D. A. Pietro. Thioridazine-induced torsade de pointes. Successful therapy with isoproterenol. JAMA : The journal of the American

- Medical Association, 249(21):2931–4, June 1983. ISSN 0098-7484. URL <http://www.ncbi.nlm.nih.gov/pubmed/6842807>.
- R. R. Kew, T. Peng, S. J. DiMartino, D. Madhavan, S. J. Weinman, D. Cheng, and E. R. Prossnitz. Undifferentiated U937 cells transfected with chemoattractant receptors: a model system to investigate chemotactic mechanisms and receptor structure/function relationships. Journal of leukocyte biology, 61(3):329–37, Mar. 1997. ISSN 0741-5400. URL <http://www.ncbi.nlm.nih.gov/pubmed/9060456>.
- M. Kim, C. Carman, and T. Springer. Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. Science, 2003. URL <http://www.sciencemag.org.libproxy.unm.edu/content/301/5640/1720.full>.
- J. Kinross-Wright. The current status of phenothiazines. JAMA : The journal of the American Medical Association, 200(6):461–4, May 1967. ISSN 0098-7484. URL <http://www.ncbi.nlm.nih.gov/pubmed/5337598>.
- S. W. Lane, D. T. Scadden, and D. G. Gilliland. The leukemic stem cell niche: current concepts and therapeutic opportunities. Blood, 114(6):1150–7, Aug. 2009. ISSN 1528-0020. doi: 10.1182/blood-2009-01-202606. URL <http://bloodjournal.hematologylibrary.org/cgi/content/full/114/6/1150>.
- D. R. Leone, K. Giza, A. Gill, B. M. Dolinski, W. Yang, S. Perper, D. M. Scott, W.-C. Lee, M. Cornebise, K. Wortham, C. Nickerson-Nutter, L. L. Chen, D. LePage, J. C. Spell, E. T. Whalley, R. C. Petter, S. P. Adams, R. R. Lobb, and R. B. Pepinsky. An assessment of the mechanistic differences between two integrin alpha 4 beta 1 inhibitors, the monoclonal antibody TA-2 and the small molecule BIO5192, in rat experimental autoimmune encephalomyelitis. The Journal of pharmacology and

- experimental therapeutics, 305(3):1150–62, June 2003. ISSN 0022-3565. doi: 10.1124/jpet.102.047332. URL <http://jpet.aspetjournals.org/cgi/content/abstract/305/3/1150>.
- B. Lewin, L. Cassimeris, V. R. Lingappa, and G. Plopper. Cells. Jones & Bartlett Learning, 2006. ISBN 0763739057. URL <http://www.amazon.com/Cells-Benjamin-Lewin/dp/0763739057>.
- T. S. Lialiaris, F. Papachristou, C. Mourelatos, and M. Simopoulou. Antineoplastic and cytogenetic effects of chlorpromazine on human lymphocytes in vitro and on Ehrlich ascites tumor cells in vivo. Anti-cancer drugs, 20(8):746–51, Sept. 2009. ISSN 1473-5741. doi: 10.1097/CAD.0b013e32832f567b. URL <http://www.ncbi.nlm.nih.gov/pubmed/19584706>.
- M. A. Lokuta, M. A. Senetar, D. A. Bennin, P. A. Nuzzi, K. T. Chan, V. L. Ott, and A. Huttenlocher. Type Iγ PIP kinase is a novel uropod component that regulates rear retraction during neutrophil chemotaxis. Molecular biology of the cell, 18(12):5069–80, Dec. 2007. ISSN 1059-1524. doi: 10.1091/mbc.E07-05-0428. URL [/pmc/articles/PMC2096574/?report=abstract](http://pmc/articles/PMC2096574/?report=abstract).
- D. Lucas, M. Battista, P. A. Shi, L. Isola, and P. S. Frenette. Mobilized hematopoietic stem cell yield depends on species-specific circadian timing. Cell stem cell, 3(4):364–6, Oct. 2008. ISSN 1875-9777. doi: 10.1016/j.stem.2008.09.004. URL <http://www.ncbi.nlm.nih.gov/pubmed/18940728>.
- B.-H. Luo, C. V. Carman, and T. A. Springer. Structural basis of integrin regulation and signaling. Annual review of immunology, 25:619–47, Jan. 2007. ISSN 0732-0582. doi: 10.1146/annurev.immunol.

25.022106.141618. URL <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1952532&tool=pmcentrez&rendertype=abstract>.

T. Matsunaga, N. Takemoto, T. Sato, R. Takimoto, I. Tanaka, A. Fujimi, T. Akiyama, H. Kuroda, Y. Kawano, M. Kobune, J. Kato, Y. Hirayama, S. Sakamaki, K. Kohda, K. Miyake, and Y. Niitsu. Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. Nature medicine, 9(9):1158–65, Sept. 2003. ISSN 1078-8956. doi: 10.1038/nm909. URL <http://www.ncbi.nlm.nih.gov/pubmed/12897778>.

J. A. McCubrey, L. S. Steelman, S. L. Abrams, F. E. Bertrand, D. E. Ludwig, J. Bäsecke, M. Libra, F. Stivala, M. Milella, A. Tafuri, P. Lunghi, A. Bonati, and A. M. Martelli. Targeting survival cascades induced by activation of Ras/Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways for effective leukemia therapy. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K., 22(4):708–22, Apr. 2008. ISSN 1476-5551. doi: 10.1038/leu.2008.27. URL <http://www.ncbi.nlm.nih.gov/pubmed/18337766>.

S. C. Mitchell. Phenothiazine: The parent molecule. Current drug targets, 7(9): 1181–9, Sept. 2006. ISSN 1873-5592. URL <http://www.ncbi.nlm.nih.gov/pubmed/17017893>.

R. E. Mudry, J. E. Fortney, T. York, B. M. Hall, and L. F. Gibson. Stromal cells regulate survival of B-lineage leukemic cells during chemotherapy. Blood, 96(5):1926–32, Sept. 2000. ISSN 0006-4971. URL <http://www.ncbi.nlm.nih.gov/pubmed/10961896>.

- B. H. Njus, A. Chigaev, A. Waller, D. Wlodek, L. Ostopovici-Halip, O. Ursu, W. Wang, T. I. Oprea, C. G. Bologna, and L. A. Sklar. Conformational mAb as a tool for integrin ligand discovery. Assay and drug development technologies, 7(5):507–15, Oct. 2009. ISSN 1557-8127. doi: 10.1089/adt.2009.0203. URL <http://www.ncbi.nlm.nih.gov/pubmed/19754304>.
- K. Noborio-Hatano, J. Kikuchi, M. Takatoku, R. Shimizu, T. Wada, M. Ueda, M. Nobuyoshi, I. Oh, K. Sato, T. Suzuki, K. Ozaki, M. Mori, T. Nagai, K. Muroi, Y. Kano, Y. Furukawa, and K. Ozawa. Bortezomib overcomes cell-adhesion-mediated drug resistance through downregulation of VLA-4 expression in multiple myeloma. Oncogene, 28(2):231–42, Jan. 2009. ISSN 1476-5594. doi: 10.1038/onc.2008.385. URL <http://dx.doi.org/10.1038/onc.2008.385>.
- M. J. Ohlow and B. Moosmann. Phenothiazine: The seven lives of pharmacology’s first lead structure. Drug discovery today, 16(3-4):119–31, Feb. 2011. ISSN 1878-5832. doi: 10.1016/j.drudis.2011.01.001. URL <http://www.ncbi.nlm.nih.gov/pubmed/21237283>.
- T. Papayannopoulou and B. Nakamoto. Peripheralization of hemopoietic progenitors in primates treated with anti-VLA4 integrin. Proceedings of the National Academy of Sciences of the United States of America, 90(20):9374–8, Oct. 1993. ISSN 0027-8424. doi: 10.1073/pnas.90.20.9374. URL <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=47570&tool=pmcentrez&rendertype=abstract>.
- P. Ramirez, M. P. Rettig, G. L. Uy, E. Deych, M. S. Holt, J. K. Ritchey, and J. F. DiPersio. BIO5192, a small molecule inhibitor of VLA-4, mobilizes hematopoietic stem and progenitor cells. Blood, 114(7):1340–3, Aug. 2009. ISSN 1528-0020.

doi: 10.1182/blood-2008-10-184721. URL <http://www.ncbi.nlm.nih.gov/pubmed/19571319>.

M. Rocco, C. Rosano, J. W. Weisel, D. A. Horita, and R. R. Hantgan. Integrin conformational regulation: uncoupling extension/tail separation from changes in the head region by a multiresolution approach. Structure (London, England : 1993), 16(6):954–64, June 2008. ISSN 0969-2126. doi: 10.1016/j.str.2008.02.019. URL <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2848535&tool=pmcentrez&rendertype=abstract>.

S. Roth and D. Zope. Phenothiazine Derivatives for Treatment of Asthma. US Patent App. 20,100/130,457, pages 1–48, 2009. URL <http://www.freepatentsonline.com/y2010/0130457.html>.

S. Roth, R. More, and B. Jameson. Intralesional treatment of psoriasis, 2005. URL <http://www.google.com/patents?hl=en&lr=&vid=USPATAPP11155450%&id=kn-aAAAAEBAJ&oi=fnd&dq=Intralesional+treatment+of+psoriasis%&printsec=abstract>.

S. Roth, C. Buck, C. Self, and G. Olson. Novel Composition for Treatment of Diseases Related to Activated Lymphocytes. US Patent App. 12/393,888, 2009. URL <http://www.google.com/patents?hl=en&lr=&vid=USPATAPP12393888&id=W67JAAAAEBAJ&oi=fnd&dq=S+ROTH+Phenothiazine+patent&printsec=abstract>.

Sigma-Aldrich. PKH26 Red Fluorescent Cell Linker Kit, PKH67 Green Fluorescent Cell Linker Kit. Technical Document, pages 1–4, 2010. ISSN 1079-7440.



- L. A. Sklar and A. Chigaev. AID 2617 - PubChem BioAssay Summary. Technical report, 2008. URL [http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=2617&loc=ea\\_ras](http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=2617&loc=ea_ras).
- S. H. Snyder, S. P. Banerjee, H. I. Yamamura, and D. Greenberg. Drugs, neurotransmitters, and schizophrenia. Science (New York, N.Y.), 184(4143):1243–53, June 1974. ISSN 0036-8075. doi: 10.1126/science.184.4143.1243. URL <http://www.ncbi.nlm.nih.gov/pubmed/17784215>.
- StemCell. Mouse Colony-Forming Cell Assays Using MethoCult  $\hat{A}$ ®. Technical Document, pages 1–25, 2005.
- S. Stübner, R. Grohmann, R. Engel, B. Bandelow, W.-D. Ludwig, G. Wagner, B. Müller-Oerlinghausen, H.-J. Möller, H. Hippus, and E. Rüter. Blood dyscrasias induced by psychotropic drugs. Pharmacopsychiatry, 37 Suppl 1: S70–8, Mar. 2004. ISSN 0176-3679. doi: 10.1055/s-2004-815513. URL <http://www.ncbi.nlm.nih.gov/pubmed/15052517>.
- G. Sudeshna and K. Parimal. Multiple non-psychiatric effects of phenothiazines: A review. European journal of pharmacology, Sept. 2010. ISSN 1879-0712. doi: 10.1016/j.ejphar.2010.08.045. URL <http://www.ncbi.nlm.nih.gov/pubmed/20828548>.
- K. Urbahns, H. Heine, and B. Junge. Use of N-substituted phenothiazines. US Patent ..., 1999. URL <http://www.google.com/patents?hl=en&lr=&vid=USPAT5861394&id=ydEYAAAAEBAJ&oi=fnd&dq=patent+phenothiazines+multiple+sclerosis&printsec=abstract>.

- A. J. Wagers, R. C. Allsopp, and I. L. Weissman. Changes in integrin expression are associated with altered homing properties of Lin(-/lo)Thy1.1(lo)Sca-1(+)-kit(+) hematopoietic stem cells following mobilization by cyclophosphamide/granulocyte colony-stimulating factor. Experimental hematology, 30(2):176–85, Feb. 2002. ISSN 0301-472X. URL <http://www.ncbi.nlm.nih.gov/pubmed/11823053>.
- D. G. Woodside and P. Vanderslice. Cell adhesion antagonists: therapeutic potential in asthma and chronic obstructive pulmonary disease. BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy, 22(2):85–100, Jan. 2008. ISSN 1173-8804. URL <http://www.ncbi.nlm.nih.gov/pubmed/18345706>.
- Z. Zeng, Y. X. Shi, I. J. Samudio, R.-y. Wang, X. Ling, O. Frolova, M. Levis, J. B. Rubin, R. R. Negrin, E. H. Estey, S. Konoplev, M. Andreeff, and M. Konopleva. Targeting the leukemia microenvironment by CXCR4 inhibition overcomes resistance to kinase inhibitors and chemotherapy in AML. Blood, 113(24):6215–24, June 2009. ISSN 1528-0020. doi: 10.1182/blood-2008-05-158311. URL <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2699240&tool=pmcentrez&rendertype=abstract>.
- Z. Zhelev, H. Ohba, R. Bakalova, V. Hadjimitova, M. Ishikawa, Y. Shinohara, and Y. Baba. Phenothiazines suppress proliferation and induce apoptosis in cultured leukemic cells without any influence on the viability of normal lymphocytes. Phenothiazines and leukemia. Cancer chemotherapy and pharmacology, 53(3):267–75, Mar. 2004. ISSN 0344-5704. doi: 10.1007/s00280-003-0738-1. URL <http://www.ncbi.nlm.nih.gov/pubmed/14663628>.
- S. H. Zigmond and J. G. Hirsch. Leukocyte locomotion and chemotaxis. New

methods for evaluation, and demonstration of a cell-derived chemotactic factor. The Journal of experimental medicine, 137(2):387–410, Feb. 1973. ISSN 0022-1007. URL <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2139498&tool=pmcentrez&rendertype=abstract>.

F. Zohren, D. Toutzaris, V. Klärner, H.-P. Hartung, B. Kieseier, and R. Haas. The monoclonal anti-VLA-4 antibody natalizumab mobilizes CD34+ hematopoietic progenitor cells in humans. Blood, 111(7):3893–5, Apr. 2008. ISSN 0006-4971. doi: 10.1182/blood-2007-10-120329. URL <http://www.ncbi.nlm.nih.gov/pubmed/18235044>.

G. Zwartz, A. Chigaev, T. Foutz, R. S. Larson, R. Posner, and L. A. Sklar. Relationship between molecular and cellular dissociation rates for VLA-4/VCAM-1 interaction in the absence of shear stress. Biophysical journal, 86(2):1243–52, Feb. 2004. ISSN 0006-3495. doi: 10.1016/S0006-3495(04)74198-3. URL <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1303916&tool=pmcentrez&rendertype=abstract>.