PHARMACOKINETICS AND P-GLYCOPROTEIN-MEDIATED TRANSPORT OF THE LEADING IMID’S IN MICE

DISSERTATION

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By

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Abstract

Lenalidomide and pomalidomide belong to a novel class of immunomodulatory (IMiD) drugs and have anti-angiogenic, anti-inflammatory, pro-erythropoietic and further anti-cancer activities. Both agents have shown promising therapeutic efficacy in multiple clinical applications, and lenalidomide has been approved by the United States Food and Drug Administration in multiple myeloma and myelodysplastic syndromes. Although many activities have been discovered, their exact mechanistic targets remain to be elucidated. Additionally, the mechanisms responsible for adverse effects are unknown. Furthermore, transporters responsible for IMiD disposition, absorbance and clearance have not yet been identified. The use of animals in this regard may prove beneficial. However, pharmacokinetic evaluations necessary for these studies are absent from the literature. Herein, we (1) validate a method for lenalidomide quantification in murine plasma and tissues and verify application of the method to those of dosed mice, (2) perform a full comprehensive pharmacokinetic evaluation of lenalidomide in mice to classify linearity, intraperitoneal and oral bioavailability from multiple doses, and tissue disposition and linearity following multiple intravenous (IV) doses and (3) perform a pharmacokinetic evaluation of pomalidomide in mice following IV and oral administration and provide evidence of IMiD transport through the ABC efflux transporter P-glycoprotein.
Dedication

This document is dedicated to my family and loved ones.
Acknowledgments

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Fields of Study

Major Field: Pharmacy

Performance in bioanalytical method development, translational research using murine models including efficacy and drug-drug interactions, and preclinical and clinical pharmacokinetics.
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Chapter 1: BACKGROUND AND INTRODUCTION

1.1 Background and Creation of IMiD’s

Immunomodulatory drugs (IMiDs) are synthesized chemotherapeutic agents undergoing current extensive clinical testing for the treatment of solid tumors, hematological and inflammatory disorders. As derivatives of thalidomide, this class of IMiDs was designed to enhance therapeutic potential of the parent drug while diminishing neurotoxicities and teratogenicity. Lenalidomide and pomalidomide were chosen as leading derivatives due to increased potency of known immunomodulatory effects\textsuperscript{1-3}. The leading IMiDs share many structural similarities with thalidomide, and their potent anti-cancer properties and diminished toxicity profile\textsuperscript{4} has led to their widespread use as chemotherapeutic agents. Lenalidomide is currently Food and Drug Administration (FDA) approved for marketing in the treatment of multiple myeloma\textsuperscript{4} and myelodysplastic syndrome (MDS) with transfusion dependent anemia\textsuperscript{5}, and is at present under investigation in a multitude of other hematological diseases including chronic lymphocytic leukemia (CLL). Lenalidomide is currently marketed by Celgene and sold under the trade name Revlimid®. Pomalidomide (trade name Actimid®) has shown therapeutic potential in MM\textsuperscript{6} and anemia associated with myelofibrosis\textsuperscript{7}. A new drug application to the FDA is expected soon based upon the efficacy observed in clinical trials to date.
As the leading synthetic IMiD derivatives maintain much of the structural homology of their precursor thalidomide, many similarities exist between activation and toxicity profiles of these compounds. For this reason, the history of thalidomide is briefly reviewed below. Thalidomide was initially indicated for pregnant females in the late 1950’s as an antiemetic and sedative until withdrawn from the market in the early 1960’s\(^8\) due to terratogenic properties\(^9\). Tragically, thousands of babies across Europe were born with malformations of the limbs known as phocomelia, later found to occur through decreased vasculogenesis caused by thalidomide targeting of angiogenic blood vessels\(^10\). Further pre-clinical testing on vasculogenesis and tumor blood vessel formation established thalidomide as an anti-angiogenic agent\(^11,12\) which led to its investigation as an anti-cancer therapeutic. The revelation of thalidomide’s approval as break-through therapy in multiple myeloma led to its ultimate recovery and exploration in a variety of other cancers. Moreover, anti-inflammatory properties of thalidomide led to investigation and ultimate approval for erythema nodosum leprosum\(^13\), a skin disorder caused by inflammation of subcutaneous adipose tissue. Thalidomide remains the frontline therapeutic treatment for this disease\(^14\). Immunomodulatory properties including inhibition of tumor necrosis factor (TNF)-alpha\(^15\) have also been observed and employed for therapeutic strategies in immune disorders. However, thalidomide association with other severe adverse effects including thrombosis, peripheral neuropathy, mood changes, constipation, fatigue, and sedation\(^16,17\) contribute to the drug’s many cautions.
The structural derivations of IMiD’s were made successful through structure activity relationship (SAR) analysis of thalidomide’s ability to inhibit tumor necrosis factor (TNF). Derivatization led to the production of active analogs up to 10,000 times more potent in lipopolysaccharide-induced peripheral blood mononuclear cell assays (PBMCs)\(^1\)\(^,\)\(^1\)\(^8\). Aside from increased potency against TNF-alpha, analogs inhibited pro-inflammatory chemokines including COX-2, IL1-beta, IL12 and IL-6 and increased anti-inflammatory cytokines such as IL-10\(^2\). As single agents in co-culture assays, activation and proliferation of NK cells and natural killer T cells was responsible for tumor cell apoptosis of hematological Raji cell lines and PC-3 cell lines, supporting use of these agents in non-Hodgkins lymphoma and prostate cancer, respectively\(^1\)\(^9\). Growth arrest and apoptosis of primary, resistant multiple myeloma cells has also been documented as a direct effect of these agents\(^2\)\(^0\). Additional drug activities similar to those of thalidomide include anti-angiogenic effects\(^2\)\(^1\) and proliferation plus co-stimulation of T-cell receptor-activated T-cells resulting in production of IL-2, IFN-gamma and upregulation of CD40 ligand\(^2\).

The leading agents chosen from this class of IMiDs include lenalidomide (CC-5013) and pomalidomide (CC-4047). Lenalidomide is an \((RS)\) alpha-(3-aminophthalimido) glutarimide with a chemical formula \(\text{C}_{13}\text{H}_{13}\text{N}_{3}\text{O}_{3}\) and a molecular mass of 259.26 g/mol. Pomalidomide is a 4-Amino-2-(2,6-dioxopiperidin-3-yl)isoindole-1,3-dione with a molecular mass 273.24 g/mol and a chemical formula \(\text{C}_{13}\text{H}_{11}\text{N}_{3}\text{O}_{4}\). The structural similarities between these two compounds and thalidomide are expressed in figure 1.
The increased potency and widespread activity of lenalidomide and pomalidomide has stimulated investigations in not only thalidomide-effective disease types, but in other hematologic malignancies and solid tumor types. TNF-alpha is identified as a therapeutic target in many autoimmune and infectious diseases due to a supportive role in disease pathogenesis, inflammation, and other clinical symptoms\textsuperscript{22-24}. Additionally, angiogenesis supports the formation, growth and metastasis of tumors and has therefore been a long pursued target of solid tumor therapeutics\textsuperscript{25}. The potential for the IMiDs in all such diseases sparked investigation in a number of these malignancies over the past decade. A review of these investigations, both pre-clinical and clinical, is included herein.

1.2. Pre-clinical Studies

Pre-clinical \textit{in vivo} murine investigations confirm many of the \textit{in vitro} observed activities and further demonstrate clinically beneficial attributes relating to the disease-supporting microenvironment. Inhibition of TNF-alpha and other pro-inflammatory cytokines have been corroborated in murine models following administration of lenalidomide\textsuperscript{26, 27} and pomalidomide\textsuperscript{28}. Both agents have displayed significant inhibition of tumor growth and metastasis\textsuperscript{29, 30} as well as anti-angiogenic properties \textit{in vivo}\textsuperscript{31-33}. Direct apoptotic activity of these IMiDs\textsuperscript{34, 35} and NK cell effects including population expansion\textsuperscript{36} and increased recruitment to the tumor site\textsuperscript{33} have also been reported. Erythropoietic stimulation and increased production of hemoglobin have been observed of pomalidomide treatment of
sickle cell mice\textsuperscript{37}. Additionally, lenalidomide has the added ability to repair the dysfunctional T-cell synapse in the mouse model of Chronic Lymphocytic Leukemia\textsuperscript{38}.

Pomalidomide is a teratogen\textsuperscript{39}, confirmed in pre-clinical studies where offspring of rabbits administered 10 to 250mg/kg daily were born with cardiac and external malformations (Pomalidomide Drug Fact Sheet, Celgene, Dec 2011). However, teratogenic effects of lenalidomide in humans have been neither proven nor disproven. Malformations similar to those observed in human ‘thalidomide babies’ have been reported in offspring of Cynomolgus monkeys who received lenalidomide during pregnancy\textsuperscript{40}. It is for this reason that lenalidomide remains characterized as pregnancy category X. A RevAssist program was initiated to ensure patient safety regarding this matter\textsuperscript{41}. Under this US program, lenalidomide can only be administered under strict evaluated supervision of registered prescribers. In women of child-bearing potential, 2 effective contraceptive methods including one very effective method and one barrier method, 2 negative pregnancy tests taken a week before and the day initiating therapy, and monthly to bi-weekly negative pregnancy tests are mandated. Additionally, a recent publication observing the presence of lenalidomide in the semen of men after four days of therapy\textsuperscript{42} demonstrates the potential exposure of a female to lenalidomide through sexual transmission. Given the capability for drug to reach a developing fetus under these circumstances, the RevAssist program additionally requires safety precautions are taken in sexually active men.
Pharmacokinetic studies of lenalidomide have been assessed in Sprague-Dawley rats following an oral dose of 50mg/kg in an aqueous dosing vehicle containing 0.5% carboxymethylcellulose and 0.25% tween 80\textsuperscript{31}. Pharmacokinetic analysis of seven plasma concentration measurements taken from the same rat (n=4) estimate an AUC of 30,000 hr*ng/ml and a half life of 2 hours. Average maximum concentration reached was 9600ng/ml at 1 hour. Pharmacokinetics have also been recently evaluated in non-human primates\textsuperscript{43}. Similarly, a dose of 20mg lenalidomide resulted in an average plasma exposure of 9 μM*h, maximum concentration of 1.4 μM at 4 hours, and a plasma half life of 5.6 hr. Although pharmacokinetic data are available in rats and monkeys, corresponding data in mice including those for pomalidomide have not been published. Nevertheless, efficacy studies have been performed in mice following common daily lenalidomide doses from 50mg/kg\textsuperscript{32, 44} to 100mg/kg\textsuperscript{26} and daily pomalidomide doses ranging from 0.5mg/kg\textsuperscript{28} and 10mg/kg\textsuperscript{37} to 50mg/kg\textsuperscript{44, 45}. As the plasma concentration achieved in mice after IMiD dosing is unreported, the clinical relevance of findings in murine studies is questionable. Therefore, pharmacokinetic properties of these agents should be thoroughly characterized.

Results from pre-clinical investigations led to the hypothesis that leading IMiD’s would be good candidates for therapy in solid tumors, inflammatory and autoimmune diseases. A brief review of the highly investigated malignancies is reported in the following paragraphs.
1.3. Background of MM, MDS and CLL

Multiple myeloma (MM) is a plasma cell malignancy affecting four per every 100,000 individuals\textsuperscript{46} and accounting for 10\% of all hematologic disorders\textsuperscript{47, 48}. Patients with MM are stratified into high- and standard risk myeloma, though both groups present with symptoms of end-organ damage including renal insufficiency, anemia, hypercalcemia and bone lesions and at least 10\% clonal plasma cellularity observed in bone marrow. Individuals diagnosed with this disease are at an increased risk of infections, however osteolytic bone lesions are the main cause of morbidity\textsuperscript{49}. A median survival time 2-3 years is observed in high risk patients and 6-7 years in standard risk disease\textsuperscript{48}. Smoldering multiple myeloma is a premalignant form of the disease where patients have not yet accrued end organ damage and has a turnover rate to MM at 10\% per year within 5 years of diagnosis\textsuperscript{50}. Staging systems for diagnosis include Durie-Salmon staging\textsuperscript{51} and the International Staging System\textsuperscript{52}, whereas the international uniform response criteria\textsuperscript{53} are in place to predict therapeutic outcome. Therapeutic strategies for low risk MM include allogeneic stem cell therapy and cyclophosphamide\textsuperscript{54} or dexamethasone with or without combination of lenalidomide for maintenance therapy and for high risk include bortezomib-based therapies\textsuperscript{55} along with allogeneic stem cell therapy. Thalidomide has shown superior activity in treating MM when in combination with dexamethasone\textsuperscript{56}. however, concerns for serious adverse effects of thalidomide such as neuropathy, deep vein thrombosis, and sedation have led away from the use of this agent as front line
therapy and towards the use of IMiDs. Despite recent advances in therapy, no curative agent exists.

The myelodysplastic syndromes (MDS) are hematopoietic myeloid malignancies which primarily manifest as peripheral blood cytopenias. MDS is more frequently diagnosed in elderly men, effecting approximately 4 per every 100,000 individuals\textsuperscript{57} and occurs as de novo MDS or as a result of prior exposure to chemotherapies or ionizing radiation therapy\textsuperscript{58}. MDS constitutes a vastly heterogeneous disease with multiple morphological classifications and an extensive range of cytogenetic abnormalities\textsuperscript{59-61}. Current classification systems, including the World Health Organization (WHO) classification\textsuperscript{59}, International Prognostics Scoring System (IPSS)\textsuperscript{62}, WHO classification-based Prognostics Scoring System (WPSS)\textsuperscript{63} and the new MD Anderson Cancer Center (MDACC) models\textsuperscript{64} have been developed for diagnosis, categorical risk assessment and prognosis. Manifestations of the disease range from asymptomatic to mild anemia in lowest grade MDS patients to neutropenia and thrombocytopenia leading to opportunistic infections in patients with higher risk MDS. Disease progression may also include transformation to acute myelocytic leukemia. Treatment options in low risk patients are aimed at improving anemia and transfusion dependency\textsuperscript{65, 66}, while high risk patients are treated with azanucleosides 5-azacitidine\textsuperscript{67} and decitabine\textsuperscript{68}, therapies similar to those used for AML including cytarabine, and allogeneic stem cell therapy\textsuperscript{69}. Although treatments have advanced in the past decade, allogeneic stem cell therapy remains the only curative treatment.
Chronic Lymphocytic Leukemia (CLL) is a hematological cancer of transformed B lymphocytes circulating throughout the bloodstream, bone marrow and lymph nodes. Monoclonal CLL B cells have genetically disrupted apoptosis pathways and anti-apoptotic influence\textsuperscript{70} causing a slowly progressed accumulation of transformed B cells which in advanced disease leads to life threatening immunosuppression and engenders patient vulnerability to opportunistic infections\textsuperscript{71-73}. According to the Surveillance, Epidemiology, and End Results (SEER) database, diagnosis of CLL is most common in elderly individuals, men with a median age of diagnosis of 72\textsuperscript{12}, and is infrequently diagnosed in subjects less than 50 years of age\textsuperscript{74, 75}. Symptoms of CLL may include lymphocytosis, adenopathy, hepatosplenomegaly, anemia and thrombocytopenia, although diagnosis of many patients succeeds a sole presentation of high WBC count\textsuperscript{76, 77}. As a means of estimating disease severity, patient survival time, and treatment option upon diagnosis, the Binet classification\textsuperscript{76} and Rai staging systems\textsuperscript{77} were developed. Treatment of asymptomatic patients in early stages has not led to increased survival advantage or quality of life; consequently, initiation of treatment is characteristically prolonged until symptoms present\textsuperscript{78}. The chemotherapies and chemoimmunotherapies currently used for treatment of CLL are not curative and several additionally impinge on the remaining healthy immune system, causing further immune suppression\textsuperscript{79}. As is recurrently the case with existing cancer therapies, resistant strains persevere after prolonged treatment, and alternate combined therapies or more aggressive therapies may be considered. CLL is not curable with currently available therapies.
1.4. Clinical Applications

1.4.1. Lenalidomide Efficacy in MM, MDS, and CLL

Lenalidomide is a therapeutic breakthrough in the treatment of MM. Original hypothesis of lenalidomide efficacy in MM were based on preclinical results of anti-angiogenic potency greater than that of thalidomide\textsuperscript{31-33}. Promising results (25% response rate) were observed in relapsed, refractory myeloma where patients were administered 15mg twice daily or 30mg/day lenalidomide for 21 days of a 28 day cycle\textsuperscript{80}. The most commonly reported toxicity was grade 3/4 myelosuppression observed in 43% of patients receiving the 15mg regimen. Little difference was observed in the median survival, time to progression and overall response rates between the two dosing regimens. Additional trials in relapsed MM demonstrated treatment with lenalidomide in combination with dexamethasone is more effective than the single agent dexamethasone\textsuperscript{81, 82}. Similar outcomes were reported in two placebo controlled studies where overall response rates of approximately 60% and complete response of approximately 15% were observed with 25mg lenalidomide plus 40mg dexamethasone\textsuperscript{83, 84}. 25mg/day lenalidomide in combination with dexamethasone has been FDA approved for refractory MM patients in June 2006. However neutropenia, thrombocytopenia and venous thromboembolism remain the main dose limiting toxicities. Lenalidomide is also currently being evaluated
and has shown promising results for newly diagnosed myeloma\textsuperscript{85-87} and as consolidation therapy after autologous bone marrow transplant (McCarthy, in review at NEJM).

Lenalidomide is approved for the treatment of transfusion dependent anemia in low or intermediate risk myelodysplastic syndrome associated with a deletion 5q abnormality\textsuperscript{88}. A phase I clinical trial of lenalidomide in transfusion-dependent, erythropoietin-refractory and poor prognosis MDS patients indicated superior response (83\%) in patients with a deletion in the long arm of chromosome 5q31.1\textsuperscript{5}. Consequent investigations refined to patients with this karyotypic abnormality revealed significant reductions in transfusion dependency where 65\% \textsuperscript{88} to 79\% \textsuperscript{89} of patients became transfusion independent. Dose-dependent cytogenetic responses reversing the cytogenetic abnormality and prolonging remission have also been reported\textsuperscript{90}, including in patients with more complex karyotypes\textsuperscript{91}. Due to life threatening dose limiting toxicities of neutropenia and thrombocytopenia\textsuperscript{89} which occur at doses lower than DLT’s observed in MM, FDA approval regulates administration at 5 and 10mg/day (Package insert).

The activity of lenalidomide against CLL is under investigation in phase II clinical trials which demonstrate therapeutic efficacy in treatment of relapsed, refractory CLL including patients harboring poor prognostic factors such as unmutated variable heavy chain and chromosomal mutations such as del(17p) or del(11q)\textsuperscript{92-94}. Clinicians and associates have reported an overall response rate (ORR) of 47\% following treatment with 25mg/day for 21 days of a 28 day cycle\textsuperscript{92}, and similar results, 38\% ORR, with a lower
dose of 10mg/day\textsuperscript{94}. Lenalidomide adverse toxicities experienced by a wide range of CLL patients (30\%-75\%) [30\% \textsuperscript{94}, 58\% \textsuperscript{92}, 75\% \textsuperscript{93}], include mild to life threatening tumor flare reaction (TFR); initiating through an unknown origin and characterized by painful enlargement of the lymph nodes and/or lymph organs associated with low grade fever, rash, and sometimes bone pain. TFR is managed by administration of corticosteroids, narcotics, or nonsteroidal anti-inflammatory agents such as ibuprofen\textsuperscript{95}. A lower dose of lenalidomide in combination with low dose-prednisone is more safe and tolerable than the initial 25mg given in early CLL trials\textsuperscript{94}. The incidence of tumor flare is not reduced with lower doses at 10mg daily oral administration versus the 25mg given in previous clinical trials, but severity of tumor flare occurrence is decreased and is more easily controlled under lower doses of lenalidomide\textsuperscript{92}. Efficacy of lenalidomide in CLL does not yet have a conclusive association with higher doses of lenalidomide or the incidence of tumor flare\textsuperscript{94}. These studies above conclude that 25mg and even 10mg of daily, orally administered lenalidomide is not an acceptable dose in this disease model and trials have resumed, initiating standard doses at 2.5mg and a TFR prophylactic with dose escalation based on patient tolerance. Interestingly, tumor flare reaction reported in CLL patients is unseen in lenalidomide treatment of MM\textsuperscript{4} or MDS\textsuperscript{5} patients.

The variability in lenalidomide disease-specific and patient-variable adverse effects and response has been highly investigated. An abstract at the American Society of Hematology annual meeting this past year administered lenalidomide to relapsed refractory CLL patients in a 3 week on, 3 week off cycle to promote TFR and investigate
correlation with patient response. Although TFR was more prevalent than in previous studies upon re-initiation of drug in cycle 2, overall response and survival was not increased. Mechanistic correlative analysis with peripheral blood versus lymph node biopsies depicted significant T cell increase and interferon-γ B-cell response specific to lymph nodes in responders. In addition, upregulation of CD40, CD54, CD86, CD95, DR5 and downregulation of CD5 and CD20 have been reported, where effects on CD5 and CD54 associate with response.

Lenalidomide has also been evaluated for the treatment of prostate cancer and refractory solid tumors. Predicted efficacy in these models was based on lenalidomide’s ability to inhibit angiogenesis essential for tumor growth and metastasis. Although increased doses of up to 35mg/day were tolerable in patients with metastatic refractory cancers, best response of lenalidomide was reported as stable disease. However, lenalidomide has demonstrated efficacy in the treatment of prostate cancer, metastatic renal cell carcinoma, and melanoma. Limitations of efficacy in select solid tumors may result from lower plasma concentrations than those indicated for efficacy in vitro. However, dose escalations above 40mg have been associated with life threatening adverse events and are accordingly seldom explored without warranted efficacy at lower doses. Efficacious outcomes have also been observed in Mantle cell lymphoma, non-Hodgkin’s lymphoma, systemic amyloidosis, and myelofibrosis with myeloid metaplasia.
These multiple clinical applications demonstrate the many activities of lenalidomide throughout a variety of cell types, including varying effects in different disease states, giving it the potential for expanded use in the treatment of multiple malignancies. Additional activities include immunomodulatory effects of B, T and NK lymphocytes, modulation of cytokine production and release, anti-angiogenic properties, alteration of tumor microenvironment, and ability to sensitize cytotoxicity to other therapies such as dexamethasone. Due to cellular activation or modulation of these many cell types, researchers have yet to corroborate the mechanism(s) of action by which lenalidomide exhibits its effects. Accordingly, the mechanisms dictating disease-specific DLTs which lead to disease-variable starting doses are additionally undetermined. Though these mechanisms remain unclear, the widespread therapeutic efficacies of lenalidomide are nonetheless evident.

1.4.2. Lenalidomide Clinical PK

Lenalidomide therapy is approved for daily administration as an oral capsule. The chemical properties of lenalidomide permit this compound to achieve significant oral bioavailability, allowing for ease of administration as oral capsules. The dosing schema of lenalidomide most commonly uses a 28 day cycle, with 21 consecutive days of a single dose/day followed by 7 days off. Administered doses are not adjusted to body weight or body surface area. There is no accumulation of drug after multiple, daily doses (Revlimid package insert). In healthy subjects, lenalidomide has a 3 to 4 hour half life
of elimination and typical maximum plasma concentrations occurring 0.6 to 1.5 hours post-dose. An average of 90% lenalidomide is cleared renally at a rate of 261 ± 30 mL/min, and 4% is excreted into feces. Data are commonly described by a 1-compartment model with mono-exponential decline of plasma concentrations.

Pharmacokinetic analyses of orally administered lenalidomide in healthy subjects conclude plasma exposures are linear to increasing doses. However, this linearity was questioned in a phase 1 trial where lenalidomide concentrations were given from 5mg up to higher doses of 40mg in patients with refractory metastatic cancers. The increase in dose caused a trend of decreasing clearance along with greater than proportional increases in AUC and Cmax. This trend was sufficiently robust to compel PK modelers to test a Michaelis-Menten equation in place of the typical equation for clearance. Although the clearance parameter was ultimately chosen, data fit well to the Michaelis-Menten model. The range of doses included in these clinical PK studies is therefore reported to fall within linear ranges. However, doses above those used in this study have not been explored and this current data suggests that higher doses may not support this linear trend.

A strong correlation between lenalidomide plasma exposure and renal function has been observed in multiple studies. Normal renal function is defined in these studies by creatinine clearance (CL\textsubscript{CR}) above 80 mL/min. In healthy subjects, lenalidomide clearance (CL) is observed around 250ml/min with 55 to 90% excreted unchanged in
the urine. In subjects with mild renal impairment (50 < \( \text{CL}_{\text{Cr}} \) < 80 mL/min), the half life of lenalidomide is increased to 9-15 hours along with a 50% reduction in clearance. In consequence, twice the lenalidomide plasma exposure is observed. Additionally, patients defined with moderate (30 ≤ \( \text{CL}_{\text{Cr}} \) < 50 mL/min) and severe (\( \text{CL}_{\text{Cr}} < 30 \) mL/min) deficiencies had respectively 38% and 43% lower lenalidomide CL. These incrementally decreasing clearance parameters in subjects with increasing degrees of renal impairment are supported by a linear trend between \( \text{CL/F} \) and \( \text{CL}_{\text{Cr}} \). As a result of this finding, modified dosing is recommended in patients with renal impairment (\( \text{CL}_{\text{Cr}} < 60 \) mL/min) (Package insert)\textsuperscript{115}.

Plasma pharmacokinetic profiles show high between subject variability in overall exposure and absorption patterns\textsuperscript{99} in which the source of variability beyond renal function has not yet been determined. The clearance of lenalidomide (90% renal) is more than 2 fold greater than renal glomerular filtration rate (the National Kidney Foundation reports an average of 90 - 120 mL/min/1.73 m\(^2\)), suggesting an active elimination component. Due to the strong dependence of PK on renal elimination and high renal clearance, a genomic irregularity affecting the function of active renal lenalidomide transporters, such as P-gp, may be involved. Other phenotypical abnormalities may also be involved but have not yet been identified. Lenalidomide is not a substrate for CYP enzymes, as phase I and phase II metabolites are not observed after \textit{in vitro} incubation with human liver microsomes\textsuperscript{116}. Hydroxylated and acetylated metabolites\textsuperscript{114} have been observed at very low abundance in plasma of dosed individuals (<5%). Hydrolysis of
lenalidomide is referred to in few publications\textsuperscript{42, 114}, however to our knowledge the structural products and activities have never been presented.

1.4.3. Pomalidomide Efficacy in MM and Other Clinical Investigations

Pomalidomide is in phase III clinical development (clinicaltrials.org), and has shown promising clinical activity in the treatment of multiple myeloma. As a single agent, dose escalations from 0.5 to 10mg pomalidomide resulted in greater than 50% overall response and complete response in 17% of relapsed or refractory MM\textsuperscript{117}. In an additional study in 60 patients with relapsed MM, 2mg pomalidomide in combination with low dose dexamethasone induced 63% overall response rate according to International Myeloma Working Group (IMWG) criteria\textsuperscript{6} including significant response in lenalidomide-refractory and bortezomib-refractory disease which were confirmed in follow-up studies\textsuperscript{118, 119}. Phase I doses reveal a maximum tolerated dose of 2mg/day on continuous schedule\textsuperscript{117} or 5mg on an alternate day cycle\textsuperscript{120}. Commonly reported toxicities are similar to lenalidomide, including anemia, thrombocytopenia, and neutropenia\textsuperscript{6}. Pulmonary toxicities observed in the above study combining pomalidomide plus low-dose dexamethasone are also believed to be an adverse consequence of pomalidomide\textsuperscript{121}.

Pomalidomide is also being tested in anemia associated with myelofibrosis and improved response rates are observed compared to lenalidomide or thalidomide therapy\textsuperscript{122, 123}. Activity of pomalidomide in myelofibrosis with associated anemia was first observed in a
trial including low dose pomalidomide in combination with prednisone\textsuperscript{7}. In a follow-up study, 37\% of patients responded using a dosing regimen of only 0.5 to 3mg/day\textsuperscript{124}. Unexpectedly, best responses were seen in the group receiving lower doses of 0.5mg. Higher incidences of adverse effects such as myelosuppression were reported with elevated doses. Nonetheless, pomalidomide has shown good overall tolerability within this disease population\textsuperscript{7, 124}. To date, the pharmaceutical sponsor of pomalidomide has seldom allowed further study of this agent outside these two disease groups.

1.4.4. Pomalidomide Clinical PK

Pharmacokinetics of 1, 2, 5, and 10mg pomalidomide at day 1 and following 4 weeks of therapy are reported in the above phase I study by Schey et al\textsuperscript{117}. On day 1, the median plasma Tmax occurred between 2.5 to 3 hours and at 4 weeks between 3 to 4 hours post dose. Pomalidomide was shown to accumulate with repeated dosing, and to a more considerable degree at higher doses. The elimination phase showed monoexponential decline starting 3 hours post dose on day 1 and 10 hours post dose on week 4, with a terminal half-life of 6 to 8 hours. Approximately 66\% of pomalidomide was recovered in the urine. Other pharmacokinetic clinical trials for pomalidomide have been initiated but no data is yet available (clinicaltrials.gov).
1.5. IMiDs and P-glycoprotein

1.5.1. Introduction to P-glycoprotein

Drug interactions commonly occur through mechanisms involving competition of transporters; many attributed to modulation of P-glycoprotein (P-gp). P-gp is an adenosine triphosphate (ATP)–dependent plasma membrane glycoprotein responsible for efflux of foreign compounds from cell cytoplasm and elimination of foreign compounds from organs or plasma, thus protecting against systemic and intracellular xenobiotic exposure\(^\text{125}\). P-gp is located in high concentrations in proximal tubules of the kidney, small intestine, liver, blood-brain barrier, uterus, testes and placenta\(^\text{126}\), as well as bronchial cells, and hematopoietic stem cells. Function of P-gp in the kidney is to transport xenobiotics from the blood into the lumen for excretion in urine\(^\text{125}\), while P-gp in the apical brush border membrane of the small intestine removes intracellular xenobiotics back to the lumen of the GI tract\(^\text{127}\). P-gp is also commonly overexpressed in the membranes of cancerous cells, including CLL B lymphocytes, and can transport intracellular therapeutics out of the cell causing multidrug resistance (MDR)\(^\text{128}\). The number of P-gp molecules expressed on the cellular surface has been shown to increase in treated patients and further increase in patients with resistant disease\(^\text{128}\).

The similarities and differences in P-gp between humans and mice have been reported. Humans have one gene encoding P-gp (\textit{ABCB1}), while mice have both \textit{mdr1a (mdr3)} and \textit{mdr1b (mdr1)} genes\(^\text{129, 130}\). Studies in mdr1 and mdr3 gene knockout models in mice
suggest the mdr3 (mdr1a) gene is responsible for P-gp expression and drug excretion in the renal proximal tubules and glomerular mesangium\textsuperscript{131-134}. The mdr1a (\texttextsubscript{-/}) murine strain exhibits increased plasma concentrations and significantly deceased elimination of multiple known P-gp modulators as compared to wild type mice\textsuperscript{133}.

1.5.2. Lenalidomide is a P-gp substrate in vitro

A recent manuscript confirms drug interaction of lenalidomide in combination with another chemotherapeutic and provides novel evidence for substrate interaction occurring through P-gp transport\textsuperscript{135}. In this clinical trial involving concomitant CCI-779 (Temsirolimus) and lenalidomide administration in the treatment of relapsed multiple myeloma, both compounds have altered PK profiles in the presence of combined therapy. Pharmacokinetic parameters reveal nearly 2 fold significantly decreased clearance, decreased Cmax and increased AUC for fixed doses of lenalidomide corresponding with an increase in dose of CCI-779, and vice versa. As CCI-779 is a known P-gp substrate\textsuperscript{136-138} and lenalidomide clearance occurs mainly through renal elimination with a suggested active elimination component, in vitro experiments testing P-gp transporter interaction were performed, revealing decreased intracellular concentrations of lenalidomide in Pgp-overexpressing cell lines which can be returned to baseline by concomitant incubation with CCI-779\textsuperscript{135}. Experiments and data analysis performed in this report provide \textit{in vitro} conclusions suggesting significant \textit{in vivo} transport of lenalidomide through P-gp.
This report has generated controversy regarding the significant role of P-gp in the \textit{in vivo} absorption and disposition of lenalidomide. Celgene has separately identified lenalidomide as weak substrate of P-gp\textsuperscript{139 2008}. To evaluate the potential for human DDIs as per FDA guidelines, a clinical evaluation was performed in combination with digoxin, a known substrate of P-gp\textsuperscript{140}. The combination resulted in 14% increase in digoxin Cmax and 8% increase in AUC; falling below the FDA guidelines for a significant interaction. Results of this study have not been published and the number of participants in each group is not reported. Moreover, any reports on the significance of P-gp transporters to lenalidomide PK are limited to this single clinical study.

In opposition to results reported from the lenalidomide-digoxin study, the clinical pharmacokinetic parameters of lenalidomide are very highly suggestive of active elimination mechanisms. Celgene itself has stated many times the likelihood of active elimination mechanisms. These claims are supported by evaluation lenalidomide clearance in combination with physiological renal elimination mechanisms and rates. Lenalidomide is excreted in urine (90%\textsuperscript{114}), where on average 105 ml/min (National Kidney Foundation) (38% of lenalidomide CL rate of 261 mL/min\textsuperscript{114}) can result from glomerular filtration. Therefore, up to 52% of lenalidomide clearance may be due to active tubular secretion. Understanding of lenalidomide as transporter substrate will allow for prediction and prevention of unintentional drug-drug interactions with combined chemotherapeutics or common, non-chemotherapeutic medications. Given the narrow therapeutic window of lenalidomide, further evaluation is warranted to determine
the significance of P-gp and other active transporters to lenalidomide absorption and disposition.

1.5.3. Pomalidomide, Thalidomide, and P-g

The interaction of thalidomide and P-gp is a disputed topic. Transport by P-gp has been observed in Madin Darby canine kidney II cell lines\textsuperscript{141}. On the contrary, other investigations have shown that no such interactions occur by showing no change in rhodamine 123 uptake in a human leukemia cell line overexpressing mdr1 and similar bidirectional transport across caco2 monolayers\textsuperscript{142}, \textsuperscript{143}. The ability of thalidomide to change expression or function of P-gp has also been explored. The sole \textit{in vivo} evidence of a thalidomide-induced change in \textit{ABCB1} gene expression lies in the experience of one multiple myeloma patient who, after 2 and 4 years of thalidomide treatment, went from 92\% P-gp positive bone marrow mononuclear cells to a complete absence thereof\textsuperscript{144}. However, \textit{in vitro} analysis of moderations in gene expression and functionality show that thalidomide neither induces nor inhibits these\textsuperscript{142}, \textsuperscript{145}.

No information is available as to whether pomalidomide interacts with P-gp.

This debate in thalidomide and IMiD interaction would be best resolved in comparative models where normal protein function can be compared to its absence, as opposed to models herein which rely on drug interactions to classify transport function. The
MDR1a/b murine model represents such a model and is employed herein to settle this long debated dilemma.

1.6. Significance

The greatest significance of IMiD therapeutics lies in the ability of these agents to treat malignancies where few therapeutic options exist. The vast activities employed by these agents allows for the potential application in treatment of many differently manifested conditions. Many of these opportunities have been realized and are currently under clinical evaluation. Still, new uses of these agents are being discovered. A mechanistic target of IMiDs and thalidomide has yet to be exposed. Toxicities documented with the use of these agents may be avoided if a mechanistic target was identified. Due to the vast activities of these agents and the need for the supporting microenvironment and cellular interactions, these studies are best performed in animal models where specific contributions can be exploited by use of knockout models. However, the lack of preclinical pharmacokinetic data in mice impinges on choosing the correct dose to represent clinically achieved drug concentrations. Additionally, drug interactions which may contribute the toxicity or therapeutic efficacy have seldom been investigated. The recent potential emergence of lenalidomide as a P-gp substrate is novel to the field, and speculates that previous reported adverse reactions may be a result of unintentional drug-drug interactions. However, this initial report was not considered valid proof of concern...
by the pharmaceutical company who markets lenalidomide\textsuperscript{146}. Unexpected drug reactions and interactions are major concerns in drug safety, as a miscalculation may be the direct cause of patient mortality. Comprehension of P-gp contribution to substrate pharmacokinetics is beneficial in understanding how to predict patient response. If transport of lenalidomide or pomalidomide is dependent on P-gp, patient variation in absorption or clearance of the drug would depend on the extent of P-gp expression or variation in MDR-1 gene controlling P-gp. Studies performed herein are aimed at determining a pharmacokinetically equivalent dose to use in murine studies of lenalidomide, performing an inclusive study on the distribution of lenalidomide to tissues and bioavailability given different administration routes and doses, and to determine both lenalidomide and pomalidomide interaction potential and contribution of the efflux transporter p-glycoprotein in mice.

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**Figure 1.1.** The structures of thalidomide and the leading IMiD derivatives lenalidomide and pomalidomide.
Chapter 2: SENSITIVE QUANTIFICATION OF LENALIDOMIDE IN MOUSE PLASMA AND TISSUES BY HPLC/APCI-TANDEM MASS SPECTROMETRY

2.1. Introduction

The increased interest in pre-clinical evaluation of the immunomodulatory drug lenalidomide within the past decade is reflective of its multiple reported mechanisms of action. Lenalidomide is FDA approved for treatment of multiple myeloma \(^1\) and transfusion dependent myelodysplastic syndrome with chromosome 5q deletion \(^2\). To date, more than 400 clinical studies have been initiated to evaluate immunomodulatory and cytoreductive benefits of lenalidomide in an array of hematological and non-hematological malignancies. Continuing trials are still revealing new therapeutic activities of this agent. Conversely, disease-specific toxicities have hindered advancement of lenalidomide in malignancies where the drug might otherwise be effective.

The mechanisms of lenalidomide activity and toxicity are not understood. Several studies have been conducted in pre-clinical \textit{in vitro} systems and in animal models to evaluate mechanistic efficacy and toxicity with lenalidomide treatment. Murine doses from 50mg/kg/day \(^3\) to 100mg/kg/day are commonly evaluated \(^4,5\). However, these studies
preclude pharmacokinetic evaluation in respective animal models. To date no pharmacokinetic data for lenalidomide in rodents has been presented in the published literature. Consequently, no data is publically available to guide efficacy and toxicity studies of lenalidomide in mouse disease models. The lack of pharmacokinetic data adds to the complexity of interpreting and extrapolating mouse efficacy and toxicity data with respect to clinical use of lenalidomide. A thorough characterization of lenalidomide disposition will therefore aid in interpretation of previous and future translational studies in mouse models.

The widespread clinical evaluation of lenalidomide throughout the past decade has led to the development of various methods for its quantification. Published assays for detection in human plasma use liquid chromatography/mass spectrometry (LCMS) coupled with liquid-liquid extraction \(^6\), solid phase extraction \(^7\), and include methods for simultaneous quantification with flavopiridol \(^8\) or dexamethasone \(^9\). LC-MS methods \(^10\) and spectrophotometric methods \(^11\) for detection in alternate matrices have also been described. These assays have been employed to evaluate pharmacokinetics of lenalidomide in patients with multiple myeloma \(^1\), metastatic refractory cancers \(^12\), relapsed or refractory acute leukemias \(^13\), and renal insufficiency \(^14\).

Herein we present a method for accurate and precise measurement of lenalidomide concentrations in mouse plasma using a standard protein precipitation, liquid-liquid extraction, and LC-MS/MS quantification. Furthermore, we apply this assay to mouse...
tissues and demonstrate its capability for assessment of lenalidomide tissue distribution studies.

2.2. Materials and Methods

2.2.1. Chemicals and Solvents

Lenalidomide powder was extracted from patient-donated commercial capsules using ethyl acetate and triethylamine as previously reported 15. Purity of extracted drug was confirmed by nuclear magnetic resonance and LC-MS. Purified lenalidomide was stored as a powder at room temperature with minimum light exposure. Stock solutions of lenalidomide and the internal standard, genistein (>98% HPLC grade, Sigma, St. Louis, MO) were prepared at 1 mM concentrations in dimethyl sulfoxide (DMSO). Different lots of pooled mouse plasmas in sodium heparin were obtained from Valley Biomedical (Winchester, VA) and Lampire Biological Laboratories (Pipersville, PA). All other materials were purchased as analytical or cell culture grade from commercial sources.

2.2.2. Mice

ICR mice were obtained from Harlan and acclimated to microisolator cages 48 hours before study initiation. Room temperature was regulated between 70°F and 72°F and kept on automatic 12-hour light/dark cycles. Mice received food and water ad libitum. All
animal care and experiments were approved and performed in compliance with the Institutional Animal Care and Use Committee guidelines.

2.2.3. Blank Tissue Collection and Sample Preparation

Untreated mice were euthanized by carbon dioxide asphyxiation followed by exsanguination via cardiac puncture. Collected blood was immediately transferred into lithium heparinized gel separator tubes (BD Microtainer, Becton, Dickson and Company, Franklin Lakes, NJ), centrifuged at room temperature for 1.5 minutes at 7,500RPM, and plasma removed and stored at -80°C until analysis. Bone marrow from hind limb femurs was collected by flushing 1mL sterile PBS through excised femurs, frozen on dry ice and stored at -80°C. Solid tissue samples including liver, lung, heart, spleen, kidney, hind limb skeletal muscle, intestine, skin, and brain were immediately excised, individually wrapped in foil, flash frozen in liquid nitrogen, and stored at -80°C. For pre-processing, solid frozen tissues were individually pulverized to a fine powder in a ceramic mortar and pestle pre-chilled on dry ice. Liquid nitrogen was added to skin samples in the mortar to facilitate solidification. Solid tissue aliquots of 90mg were allocated and returned to -80°C for storage.

2.2.4. Plasma and Tissue Sample Processing

Plasma (both purchased and freshly obtained) and bone marrow samples were thawed at room temperature, vortex mixed, and briefly centrifuged. Portions of each sample (90μL) were then transferred to a clean microcentrifuge tube. Aliquots of solid tissue samples (90
mg) were thawed at room temperature, diluted with 100μL water and vortex mixed every 5 minutes for 20 minutes. For calibration standard and quality control (QC) validation samples, 10μL of 10X lenalidomide solutions in DMSO were added to each sample to achieve desired drug concentrations between 0.3nM and 30μM. After mixing, lenalidomide was extracted from plasma and tissue samples by addition of 4°C acetonitrile containing 200nM genistein. Samples were vortex mixed every 5 minutes at room temperature for 20 minutes, centrifuged at 4°C for 10min, and supernatant removed and evaporated in a speed vacuum for 6 hours. Dried samples were reconstituted in 120μL water containing 0.1% formic acid, vortex mixed and centrifuged as in previous steps, and 100μl of supernatant transferred to auto-sampler vials for quantification.

2.2.5. Recovery, Matrix Effect and Process Efficiency

Plasma and tissues were harvested from untreated mice and pre-processed as described above. Matrices (A), including acetonitrile as neat solution (B), were first extracted and spiked after extraction at triplicate low (60nM) and high (6μM) reconstitution concentrations of lenalidomide containing a single known amount of genistein (6 samples per set). Additional sets of blank plasma and tissue samples (C) were spiked at low and high concentrations before extraction. Calculations were performed separately for lenalidomide and internal standard using observed chromatographic peak areas. Matrix effect (%) was calculated as A/B x 100; process efficiency (%) as C/B x 100; and recovery (%) as C/A x 100.
2.2.6. LC-MS/MS

Lenalidomide was detected using a modified version of a validated and sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) assay. Samples (20μl) were injected into an Accela (Thermo, Waltham, MA) ultra high performance liquid chromatography system using a flow gradient of water (solvent A) and acetonitrile (ACN) (solvent B) both containing 0.1% Formic Acid. Separation was achieved by reverse phase chromatography using a Zorbax (Agilent) C-18 extended column with a Metaguard C-18 guard column. The 6 minute method initiated with mobile phase composition at 100% A with a 0.3 minute gradient to 90% B, was held at these conditions from 0.4 min to 2.7 min, and was followed by a 0.3min return to initial conditions for column equilibration for the remaining run time. A TSQ Quantum Discovery Max mass spectrometer (Thermo) equipped with an atmospheric pressure chemical ionization probe in positive ion mode was used for MS/MS analysis. Ion transitions monitored for lenalidomide and genistein were 260.06>149.10 and 271.09>152.90, respectively. All other mass spectrometer settings were as previously reported.

2.2.7. Plasma Pharmacokinetic Pilot Study

Lenalidomide dosing solution was prepared at a concentration of 100μg/ml in PBS containing 0.1% HCL. Following complete visible dissolution, the solution pH was adjusted to 7.4 using NaOH and sterile filtered using a 0.22μm Steriflip (Millipore, Billerica, MA) filter. A single dose of 1.5mg/kg (normalized to body weight at 5μl/g of mouse) was administered as an intravenous bolus injection via the tail vein. Plasma was
extracted from blood samples collected at 2, 10, 20, 40 minutes and 1, 1.5, 3, 5, 7, 10, and 18 hours using the method described above.

2.2.8. In Vivo Tissue Pilot Study

ICR mice were administered a single 1.5mg/kg IV bolus injection of a 300μg/ml lenalidomide dosing solution prepared as in the PK study. Plasma, brain, liver, muscle, heart, spleen, lung, kidney, bone marrow, skin and intestine were collected at 10, 45, and 90 minutes post dose from n=3 mice per time point (9 mice). Tissues were pre-processed and prepared according to above methods using 100mg homogenate.

Bone marrow collection required flushing of both femurs with 1mL PBS. Due to low quantities of bone marrow obtainable from mice, an accurate and direct mass for each bone marrow sample could not be achieved. Therefore an average bone marrow mass was determined to represent all samples. This was achieved by weighing cut femur bones (n=5) pre- and post- flushing with PBS, followed by washing with ethanol then drying at room temperature. An average of 26.08 ± 4.7mg bone marrow sample was established and applied to calculate the concentration of all unknown bone marrow samples.

2.3. Results and Discussion

2.3.1. Assay Conditions
The LC-MS/MS method was based on the ultra-sensitive method described by Liu et al.\textsuperscript{8}. The method was modified for murine studies using 100μL sample volumes and the altered method procedures as described above. ACN and ethyl acetate (EtAc) were evaluated for extraction efficiency of lenalidomide from tissue. ACN achieved superior extraction from tissues and was selected as the extraction solvent for plasma and all tissues (data not shown).

2.3.2. Selectivity

No interfering peaks were observed in blank plasma with or without the internal standard. Representative ion chromatograms from blank plasma and from plasma spiked both with lenalidomide at the lower limit of quantification (LLOQ) of 0.3nM and genistein are displayed in Figure 2.1A-B. Similarly, chromatograms from blank bone marrow and bone marrow spiked with 1nM lenalidomide and genistein are displayed in Figure 2.1C-D as examples of selectivity in tissues. Increased background signals were observed in some tissues, including intestine which had the highest observed background.

2.3.3. Linearity and Sensitivity

To determine the linear range for quantification, mouse plasma and tissues were harvested from untreated mice and spiked with lenalidomide and internal standard. Lenalidomide was linearly quantifiable in plasma from a lower limit of quantification (LLOQ) of 0.3nM up to 30μM. Response ratios plotted against target concentrations display varying slopes and acceptable linearity (Figure 2.2) up to 30μM in all tissues.
Linearity extends to 0.3nM in brain, muscle and heart, 1nM in lung and bone marrow, 3nM in skin, 10nM in liver, kidney, and spleen, and 100nM in intestine.

2.3.4. Accuracy and Precision
Varied sources of mouse plasma including freshly harvested plasma from untreated mice and pooled mouse plasma purchased from 2 suppliers were used in calibration curves to assess method accuracy and precision. No differences were observed between the three sources of plasma (data not shown). Inter-day accuracy and precision from 5 plasma standard curves are shown in Table 2.1.

2.3.5. Recovery, Matrix Effect and Process Efficiency
Triplicate low (60nM) and high (6μM) concentration samples were analyzed and evaluated as described in materials and methods. Mean data are tabled separately for lenalidomide (Table 2.2) and genistein (Table 2.3). Mouse plasma demonstrated good recovery, percent efficiency and absence of matrix effect for lenalidomide. The overall process efficiency was satisfactory in all tissues except intestine (5.55%), ranged from 30-50% in kidney, liver, lung and spleen, and was higher in other tissues. Low intestine response ratio was a consequence of matrix suppression of lenalidomide ion intensity and matrix enhancement of internal standard.

2.3.6. Pharmacokinetics
The method was applied in a pilot pharmacokinetic study where mice received 1.5mg/kg lenalidomide via IV bolus injection. Plasma concentrations fell within the linear range of the method and were plotted against time in Figure 2.3. A detectable “Cmax” of 12.5μg/mL was achieved 2 minutes post dose. Drug concentrations fell below the detectable limit of quantification at 3 hours post injection.

Tissues collected from in vivo studies were also evaluated. Tissues and plasma from 3 mice were collected 10m, 45m, and 1.5hr after a single 1.5mg/kg IV bolus dose of lenalidomide. Pilot tissue concentration-time profiles (Figure 2.4) show comparative, decreasing concentrations throughout the time course studied. This pilot data suggests highest concentrations are achieved in plasma, and tissues range in concentration (highest to lowest) in the order of spleen, lung, liver, bone marrow, hind limb muscle, kidney, and heart. Lenalidomide could not be detected in brain at this dosing level, presumably due to efflux by P-gp transporters in the blood brain barrier 16. Intestine samples were not quantified as the response ratio detected in assay validation studies was very low. Furthermore, skin samples were also not quantified as the method for sample preparation of this tissue is not easily applicable to large sample numbers.

2.4. Conclusion
A validated method for the detection and quantification of lenalidomide in mouse plasma and tissues is presented. Analytical accuracies, precisions and process efficiencies are within an acceptable range for all tissues excluding intestine. The pilot application of this method to plasma and tissues harvested from dosed mice confirms its merit in future pharmacokinetic and disposition studies.

2.5. References


Figure 2.1. Representative chromatograms of lenalidomide

(A) blank plasma; (B) plasma LLOQ, 0.3nM; (C) blank bone marrow; (D) bone marrow 1nM.
Figure 2.2. Representative standard calibration curves of 0.3nM to 30μM lenalidomide in plasma and tissues.

A) Response ratio vs. nominal concentration. B) Slopes of the standard lines for neat and tissue matrixes. Calculated from the equation $y = mx + b$ with $1/Y$ weighting.
Table 2.1. Plasma inter-batch accuracy and precision.

Data are mean % difference from nominal (Accuracy) and coefficient of variation (Precision, calculated as \[\frac{(\text{Standard deviation of the observed concentrations})}{(\text{mean observed concentration})}] \times 100) from n = 5 standard curves in separate batches. NV, nominal value.

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<th>NV (nM)</th>
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<tbody>
<tr>
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<td>Tissue</td>
<td>% Recovery Low (60nM)</td>
<td>% Recovery High (6μM)</td>
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<td>----------------------</td>
<td>----------------------</td>
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<tr>
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<tr>
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Table 2.2. Summary of lenalidomide recovery, matrix effect and process efficiency.

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<th>% Recovery Low (60nM)</th>
<th>% Recovery High (6μM)</th>
<th>% Matrix Effect Low (60nM)</th>
<th>% Matrix Effect High (6μM)</th>
<th>% Process Efficiency Low (60nM)</th>
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Table 2.3. Summary of genistein recovery, matrix effect and process efficiency.

(n = 3)
**Figure 2.3.** Pilot plasma pharmacokinetics.

Plasma concentration-time plot for lenalidomide pilot study in mice administered 1.5 mg/kg IV bolus injection.
Figure 2.4. Pilot tissue pharmacokinetics.

Tissue concentration-time plot for lenalidomide in plasma and tissues. Data is presented as nanograms lenalidomide per gram of tissue (ng/g) or per milliliter of plasma (ng/mL).

Error bars represent standard deviation. n=3
Chapter 3: PHARMACOKINETICS AND TISSUE DISPOSITION OF LENALIDOMIDE IN MICE

3.1. Introduction

Lenalidomide is a synthetic immunomodulatory drug (IMiD) analog of the anti-angiogenic drug, thalidomide. Although initially selected as a lead compound for potent inhibition of tumor necrosis factor-alpha, lenalidomide exhibits further activities including anti-angiogenic properties, increased erythropoiesis, direct induction of tumor apoptosis, modulation of pro-survival and inflammatory cytokines, an increase in immune effector cell response and synergistic effects in combination therapies. On account of this extensive activity profile, exploration of drug efficacy across multiple disease states has been under evaluation since the drug’s clinical release in the early 2000’s. In the last decade lenalidomide has met FDA approval for multiple myeloma (MM) and myelodysplastic syndrome (MDS). It also demonstrates clinical activity in systemic amyloidosis, chronic lymphocytic leukemia (CLL), mantle cell lymphoma, myelofibrosis with myeloid metaplasia, non-Hodgkin’s lymphoma and continues to be investigated for treatment of other cancers and hematological conditions. This widespread anti-cancer response is believed to result from a unique
combination of the drug’s many activities. However, the mechanism responsible for induction of these events remains unclear.

Disease-specific toxicity profiles and potentially life-threatening adverse events associated with lenalidomide treatment are responsible for considerable diversity in starting doses. In the majority of indications, neutropenia and thrombocytopenia are the most frequent dose limiting toxicities \(^{11, 21, 22}\). However, increased severity of these adverse events yields a reduced 10 mg dose in MDS compared to the standard 25mg dose in MM \(^{11}\). In contrast to lower grade myelosuppression, patients with MM are at greater risk for thromboembolic events \(^{23, 24}\) as compared to MDS patients. The conventional 25mg starting dose in clinical trials is also poorly tolerated in other hematological malignancies such as systemic amyloidosis \(^{12}\) and CLL \(^{25}\). Tumor flare reaction appears to be exclusive to CLL and remains poorly understood mechanistically. Typically associated with a cytokine release syndrome, the extreme severity of lenalidomide’s response in CLL is accountable for the significant limitations in starting dose for current clinical trials. The mechanistic differences responsible for these disease-specific toxicities remain unclear.

The unknown mechanism of action and ill-defined source of adverse effects in these clinical evaluations reflect an unmet need for further translational research. Efficacy and toxicity of lenalidomide have been previously reported in mice \(^{9, 26, 27}\). However, to date no pharmacokinetic data has been presented in mice. It is therefore unclear if the doses
used in these previous studies produce systemic concentrations that are relevant to those observed in the clinic. In support of ongoing pre-clinical and translational evaluation of lenalidomide, we report a comprehensive pharmacokinetic characterization of lenalidomide in plasma and tissues of mice.

3.2. Materials and Methods

3.2.1. Chemicals and Solvents
Stock lenalidomide powder was purified from commercial capsules in a method previously reported\(^\text{25}\) and stored at room temperature with minimum light exposure. Dosing solutions were prepared by adding lenalidomide to the appropriate volume of sterile phosphate buffered saline (PBS) containing 1% hydrochloric acid (HCL). Following complete drug dissolution, the pH of this preparation was adjusted to 7.0 - 7.6 using sodium hydroxide and sterile filtered using a 0.22μm Steriflip filter (Millipore, Billerica, MA) prior to dosing. Genistein (>98% HPLC grade) was obtained from Sigma (St. Louis, MO). All other materials were purchased as analytical or cell culture grade from commercial sources.

3.2.2. Mice
Imprinting Control Region (ICR) mice 8 to 10 weeks of age were obtained from Harlan Laboratories (Indianapolis, IN) and acclimated to the animal care facilities for a
minimum of 48 hours before study initiation. To minimize exogenous exposure, mice were housed in microinsulator cages. Room temperature was regulated between 70°F and 72°F, and automatic 12-hour light/dark cycles were maintained. Mice received food and water ad libitum, with the exception of orally administered mice which had food removed on the evening prior to dosing and withheld until 3 hours post dosing. All animal care and experiments were approved and performed in compliance with the Institutional Animal Care and Use Committee guidelines.

3.2.3. Dosing

Mice were administered sterile preparations of lenalidomide normalized to body weight for appropriate dosing concentrations. Intravenously (IV) dosed animals received drug by bolus tail vein injections, and extravascularly dosed mice received drug by bolus intraperitoneal injections (IP) or oral gavage (PO). With the exception of the range finding studies, dosing volume was normalized to body weight (5 µL per gram) so that mice received between 150 to 200 µL.

3.2.4. Range Finding Study

Lenalidomide solubility was poor at 3.5 mg/mL and above in PBS containing 1% HCL. Mice were administered lenalidomide dosing concentrations up to 3 mg/mL at the maximum volume approved in the animal use protocol guidelines. Volumes were administered at 5 µL/mg IV, 7.5 µL/mg IP, and 15 µL/mg PO, not to exceed 200 µL IV, 300 µL IP, and 600 µL PO. Single mice were evaluated at escalating doses of 3, 10, and
15 mg/kg IV; 4.5, 15, and 22.5 mg/kg IP; and 9, 30, and 45 mg/kg PO. Additional mice (n = 4) were evaluated at the maximum dose achievable by volume and solubility of dosing solution. All mice were monitored closely for one hour, and re-evaluated for toxicities 3 hr, 6 hr, and 24 hr post dose.

3.2.5. Sampling and Tissue Preparation

Mice were sacrificed by carbon dioxide asphyxiation followed by exsanguination at time points 2 minutes to 8 hours post dose. Blood was collected by cardiac puncture into lithium heparinized plasma separator tubes (BD Microtainer, Becton, Dickson and Company, Franklin Lakes, NJ), centrifuged at room temperature for 1.5 minutes at 4,200 RCF, and plasma removed and stored at -80°C until analysis. Bone marrow from hind limb femurs was flushed into 1mL of sterile PBS and immediately placed on dry ice until transferring to -80°C freezer for storage. Liver, lung, heart, spleen, kidney, hind limb muscle, and brain were individually wrapped in foil and flash frozen in liquid nitrogen. Solid tissues were individually pulverized via mortar and pestle on dry ice, weighed in aliquots and stored at -80°C until processing.

3.2.6. Sample Processing

Sample processing and quantitative analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS) is described elsewhere (manuscript submitted). Briefly, frozen samples were removed from storage and warmed to room temperature prior to processing. Standard curves were prepared in blank mouse plasma, neat solution (100%
acetonitrile) or homogenized tissues. After brief vortex mixing and centrifugation, plasma and bone marrow sample aliquots were taken for processing and quantification. Solid tissue homogenates were incubated in an equal volume of water for 20 minutes prior to extraction. Drug was extracted from plasma and tissues via protein precipitation with 4°C acetonitrile containing 200 nM genistein as internal standard. Supernatant was removed and evaporated under vacuum for 6 hours. Samples were reconstituted in HPLC grade water containing 0.1% formic acid and transferred to autosampler vials. Extract impurities were separated by an Extended C-18 column (Agilent, 50 mm x 0.5 mm, 3 μm particle size) using an ultra high performance Accela liquid chromatography system (Thermo, Waltham, MA) and a flow gradient of water and acetonitrile both containing 0.1% formic acid. Analytes were ionized with atmospheric pressure chemical ionization, and fragment ions were detected on a TSQ Quantum Discovery Max triple quadrupole mass spectrometer system (Thermo).

3.2.7. Pharmacokinetic Analysis

Pharmacokinetic (PK) parameter estimates were generated from plasma and tissues using WinNonlin Professional version 5.2.1 (Pharsight Corporation, Mountain View, CA). Noncompartmental analysis was performed using a linear up/log down approach for area under the concentration-time curve (AUC) calculation and uniform weighting for terminal phase regression. Compartmental model fitting was completed by evaluating one-, two- and three-compartment models with uniform, 1/Y, 1/Y², and 1/Y_{hat} weighting
schemes. Model discrimination was based on visual fit, weighted residuals, individual parameter coefficients of variation and Akaike’s information criterion.

3.3. Results

3.3.1. Range-finding study
Doses administered to mice were limited by solubility of lenalidomide in the PBS dosing solution. Maximum achievable doses of up to 15 mg/kg, 22.5 mg/kg, and 45 mg/kg were given through IV, IP and PO routes, respectively. IV administration was fatal in one mouse immediately following the 15 mg/kg bolus injection. No other fatalities or observable toxicities were reported at maximum doses. Due to solubility limitations which prevented further dose escalation, a maximum tolerated dose was not established.

3.3.2. Plasma Pharmacokinetics: IV dosing
As the range finding study resulted in mortality at the 15 mg/kg maximum dose in IV dose-toxicity studies, upper-limit doses of 10 mg/kg were utilized in pharmacokinetic studies regardless of administration route. Dose proportionality was assessed following IV bolus doses of 0.5, 1.5, 5 and 10 mg normalized to body weight. Plasma concentration versus time plots are shown in figure 3.1, and estimated non-compartmental calculated parameters are listed in table 3.1. Maximum observed plasma concentrations (Cmax) at 2 minutes post dose ranged from 0.28 µg/mL to 17.75 µg/mL across the 4 evaluated doses.
For data modeling, all final compartmental best fit IV models (*figure 3.2*) employed a 1/Y_{hat} weighting scheme. Following IV doses of 1.5 mg/kg and greater, lenalidomide concentrations decreased triexponentially, with average compartmental alpha, beta and gamma half lives of 0.6, 13.4, and 85.1 minutes, respectively (*table 3.2*). The 0.5 mg/kg time-concentration profile produced a biphasic decline with alpha and beta half lives of 5.2 and 50.6 minutes, respectively. Non-compartmental analysis resulted in nonlinear AUC parameter estimates ranging from 7.8 to 226 µg*min*mL^{-1} over the chosen dosing range. Plasma concentrations and AUC became saturated at the higher doses as displayed in *figure 3.3*. The data indicate an approximate 17-fold increase in AUC while the dose increased 3-fold from 0.5 to 1.5 mg/kg. Following doses of 1.5 mg/kg and greater, the increase in plasma AUC is less than proportional, with an average fold increase of 0.51 per unit fold increase in dose.

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### 3.3.3. Plasma Pharmacokinetics: Extravascular dosing

Bioavailability was assessed for IP and PO administration routes following doses of 0.5 and 10 mg/kg. Concentration-time plots and resulting compartmental fit models are shown in *figure 3.4* and *figure 3.5*, respectively. Maximum observed concentrations following 0.5 and 10 mg/kg were 0.246 and 8.343 µg/mL for IP administration, and 0.092 and 2.447 µg/mL for PO administration (*table 3.1*). Observed time at maximum concentration (Tmax) occurred at 10 minutes for both IP doses and 20 and 40 minutes for 0.5 and 10 mg/kg oral doses, respectively. Although IP dosing achieved a Cmax 2.7 to
4.2 fold greater than oral administration, orally dosed mice sustained drug concentrations above 1.00 µg/mL 1.5 hours post a 10 mg/kg dose compared to 0.39 µg/mL for mice receiving IP doses.

IP and oral compartmental data were weighted as 1/Y. Apparent absorption rate constants (Ka) were higher for IP than PO routes, with a fold difference of 7.3 and 2.9 for 0.5 and 10 mg/kg, respectively (table 3.2). Concentration-time data indicated monophasic and biphasic decline following PO and IP administration, respectively. Compartmental estimates for Tmax were 10 minutes and 27 minutes, respectively, regardless of dose. Systemic bioavailability determined from AUC estimates were 69% and 75% for PO doses versus 90% and 105% for IP doses of 0.5 and 10 mg/kg, respectively. These averaged to PO and IP bioavailabilities of 72% and 98%, respectively.

3.3.4. Tissue Pharmacokinetics

Using our LC-MS/MS method for tissue quantification (described elsewhere), we compared the levels of lenalidomide in a variety of autologous tissues collected from mice dosed with 0.5, 1.5, 5, and 10 mg/kg IV lenalidomide. The resulting concentration-time profiles from heart, lung, liver, muscle, spleen, bone marrow, brain, and kidney tissues are displayed in figure 3.6 a-d. Initial concentrations expressed by 2 minute time points in order from most prominent highest to lowest concentration are lung, liver, heart and spleen. Following a dose of 10 mg/kg, these reported 2 minute concentrations achieve 15, 5, 2.4 and 1.3 µg/g, respectively. At 45 minutes post the 10 mg/kg dose the
highest concentrations are found in lung, liver, and spleen, with concentrations of 268 to 612 ng/g. Less than 15 ng/g remains in each tissue after 8 hours.

Tissues with the greatest lenalidomide exposure were lung, spleen and liver, as reported by non-compartmental AUC values in **figure 3.7**. In contrast, lenalidomide could not be detected in brain tissues following 0.5 and 1.5 mg/kg doses, but was quantifiable at low concentrations following 5 and 10 mg/kg injections. An absorption phase was observed in spleen at all administered dosing levels and in muscle only with the 10 mg/kg dose. Observed peak concentrations in spleen occurred at 10 minutes post dose and were approximately 2-fold higher than concentrations at the earliest 2 min time point. An absorption profile was also observed in muscle in the 10 mg/kg dose group with a Tmax of 10 minutes. At all lower doses, muscle concentrations remained relatively constant between 2 and 20 minutes before descending.

Given a single dose, the distribution of lenalidomide varies for each tissue. Additionally, lenalidomide distribution to each tissue type varies over the dose range evaluated. These tissue nonlinearities are observed when tissue AUC’s are plotted against a linear dosing scale (**figure 3.8**) and when lenalidomide distribution to tissues is expressed as the ratio of tissue exposure/plasma exposure (**figure 3.9**). The ratio of lenalidomide distributed to tissues is lowest with the 1.5 mg/kg dose.
3.4. Discussion and Conclusions

Clinical efficacy of lenalidomide in multiple malignancies results from its many activities spanning from anti-angiogenic and hematological responses to enhanced erythropoiesis. Extensive clinical evaluations are underway, but dose dependent and disease specific toxicities remain a challenge. Further translational research in animal models is required to enhance our understanding of lenalidomide mechanisms of action and toxicity. With limited published pharmacokinetic data in animals, we sought to provide a thorough characterization of lenalidomide pharmacokinetics in mice to guide ongoing and future lenalidomide investigations in murine disease models.

A recently developed sensitive assay for quantification of lenalidomide in mouse plasma and tissues was implemented in the present study (manuscript submitted). We conclude lenalidomide has nonlinear kinetics in ICR mice over a dosing range of 0.5 to 10 mg/kg. Average systemic bioavailability of lenalidomide was 72% after oral administration and 98% after IP administration. Lenalidomide was distributed to all analyzed tissues, although levels were undetectable in brain with the 0.5 and 1.5 mg/kg doses. Tissue-to-plasma ratios calculated at each dose further demonstrate the nonlinearity observed in plasma.

The observed nonlinearity is suggestive that increases in mouse doses above 10 mg/kg will result in under-proportional increases in plasma exposure. Thus, previous pre-clinical
mouse doses exploring 50 mg/kg\textsuperscript{9} and 100 mg/kg\textsuperscript{27,28} likely do not reflect proportional increases in plasma exposure. Although dose escalating pharmacokinetic clinical investigations have generally concluded dose proportionality in AUC, the linearity in humans has been previously challenged\textsuperscript{29}. Nevertheless, low doses in mice result in similar plasma concentrations, high oral bioavailability, and monoexponential decline as seen in humans. When comparing mouse pharmacokinetic data to that in humans, a 1.5 mg/kg IV dose in mice produces a non-compartmental AUC (2130 h*ng/mL) most similar to lenalidomide plasma exposure following a 25 mg dose in human patients (AUC = 2154 h*ng/mL)\textsuperscript{11}. Future doses used for translational studies in mice should consider this data while choosing clinically relevant doses.

Compartmentally estimated volumes of distribution of lenalidomide are greater than the volume of plasma and extracellular compartment in the mouse, indicating that lenalidomide has substantial distribution to tissues. Quantification of lenalidomide in 8 tissues from IV dosed mice confirms this, with dissimilar kinetics in spleen and brain tissues. Lenalidomide distribution to spleen includes an absorption phase with a Tmax of 10 minutes in all doses tested. This accumulation may be explained by the presence of uptake transporters or high affinity binding to biomolecules expressed specifically in the spleen. To our knowledge these possibilities have not been reported previously and should be further explored. This preferential distribution to spleen may be relevant for lenalidomide efficacy and toxicity mechanisms in lymphoid organs.
Lenalidomide distribution to brain is detectable in our study only at doses of 5mg/kg and above. With an assay lower limit of quantification of 0.3 ng/g in brain tissue, the data suggest relatively poor penetration into brain. This is not surprising given recent evidence that lenalidomide is a substrate of the efflux transporter, P-glycoprotein. This active transporter mediates xenobiotic removal from the brain via the blood brain barrier which would reduce overall lenalidomide uptake in the central nervous system (CNS). A recent publication exploring distribution of lenalidomide in nonhuman primates determined 11% exposure in cerebrospinal fluid following a dose of 20 mg. Similarly, we report lenalidomide brain exposures following 5 and 10mg/kg in mice are approximately 0.9 and 2.3% of that observed in plasma. These results may explain why clinical neurotoxicities are minimal and reported in few patients relative to thalidomide. Furthermore, co-administration of agents that may increase lenalidomide penetration (e.g. through inhibition of P-gp) may increase the prevalence of neurotoxicities observed in the clinic. While studies are being pursued to evaluate lenalidomide efficacy in cancers of the CNS and other neurological disorders, the relatively low lenalidomide CNS penetration may become a limiting factor. Alternatively, P-gp inhibition may be a strategy to increase lenalidomide distribution at the target site in CNS diseases.

This work provides data for plasma pharmacokinetics and tissue distribution of lenalidomide in mice at clinically relevant doses. Intraperitoneal and oral administration routes demonstrate good bioavailability and provide acceptable dosing routes in mice. These studies can be used to estimate concentrations achieved at relevant sites of drug
action when lenalidomide efficacy studies are performed in murine models. Considerations of nonlinearity and selective tissue distribution reported herein may aid in selection of doses with greater clinical relevance to better enable interspecies extrapolation of results in continued clinical and translational development of lenalidomide.

3.5. References


8. Zhu D, Corral LG, Fleming YW, Stein B: Immunomodulatory drugs Revlimid (lenalidomide) and CC-4047 induce apoptosis of both hematological and solid tumor cells through NK cell activation, Cancer Immunol Immunother 2008, 57:1849-1859


MA: Combination therapy with lenalidomide plus dexamethasone (Rev/Dex) for newly diagnosed myeloma, Blood 2005, 106:4050-4053


Figure 3.1. Plasma concentration vs. time plots for intravenous doses.

Error bars represent standard deviation from n=5 mice.
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<th>Dose (mg/kg)</th>
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<th>Cmax (ng/ml)</th>
<th>t₁/₂ (min)</th>
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<th>Vz (L/kg)</th>
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Table 3.1. Non-compartmental parameter estimates for IV, IP, and PO dosing routes.

IV, intravenous; IP, intraperitoneal; and PO, oral gavage; F, bioavailability calculated as [100 x (AUC<sub>extravascular</sub> * Dose<sub>IV</sub>)/(AUC<sub>IV</sub> * Dose<sub>extravascular</sub>)]. AUC, area under the concentration-time curve; Cmax, maximum observed concentration; t₁/₂, terminal phase half-life. CL (clearance) and Vz (volume of distribution) were estimated as CL/F and Vz/F then multiplied by the bioavailability calculated from non-compartmental AUCs.
Figure 3.2. IV plasma pharmacokinetic compartmental model parameter estimates.

Data from the 0.5mg/kg dose was fit to a two-compartment model. Data from other doses were fit to three-compartment models. Data was weighted as 1/Yhat.
Table 3.2. Compartmental parameter estimates for IV, IP, and PO dosing routes.

IV, intravenous; IP, intraperitoneal; and PO, oral gavage; AUC, area under the estimated concentration-time curve; Cmax, maximum estimated concentration; $t_{1/2}$, half-life for each phase; Ke, estimated elimination rate constant; Ka, estimated absorption rate constant. V, volume of distribution reported as Vss (steady state) for intravenous routes and V/F output from IP and PO models multiplied by compartmentally estimated F. For extravascular doses CL is represented as CL/F output multiplied by F. F = bioavailability computed as $[100 \times (\text{AUC}_{\text{extravascular}} \times \text{Dose}_{\text{IV}})/(\text{AUC}_{\text{IV}} \times \text{Dose}_{\text{extravascular}})]$.

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<th>Dose (mg/kg)</th>
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**Figure 3.3.** Plasma compartmental AUC estimates versus intravenous dose.

AUC, area under the concentration-time curve.
Figure 3.4. Plasma concentration vs. time plots for extravascular doses.

Error bars represent standard deviation from n=5 mice.
Figure 3.5. Plasma pharmacokinetics and compartmental model estimates for extravascular doses.

Extravascular doses include intraperitoneally (IP) and orally (PO) administered doses. Oral and intraperitoneal data were fit to one-compartment and two-compartment models, respectively. All data was weighted as 1/Y.
Figure 3.6. Representative tissue and plasma concentration vs. time data.

a) 0.5mg/kg;  b) 1.5mg/kg;  c) 0.5mg/kg; and  d) 10mg/kg doses. Data points are means from n=5 animals. Y-axis units for plasma are (ng/mL).

Continued…
Continued from Figure 3.6.

c)

Tissue 5mg/kg

Time (min)

Concentration (ng/g)

- Plasma Avg
- Brain Avg
- Liver Avg
- Mscl Avg
- Heart Avg
- Spln Avg
- Lung Avg
- Kidney Avg

d)

Tissue 10mg/kg

Time (min)

Concentration (ng/g)

- Plasma Avg
- Brain Avg
- Liver Avg
- Mscl Avg
- Heart Avg
- Spln Avg
- Lung Avg
- Kidney Avg
Figure 3.7. Non-compartmental tissue and plasma AUC estimates displayed as categorical bar plots.

Units for plasma are (min*µg/ml).
Figure 3.8. Non-compartmental tissue and plasma AUC estimates displayed as scatter plots.

Units for plasma are (min*μg/ml).
Figure 3.9. Non-compartmental tissue and plasma AUC estimates displayed as tissue-to-plasma ratios versus dose.

Units for plasma are (min*μg/ml).
Chapter 4: LENALIDOMIDE AND POMALIDOMIDE ARE SUBSTRATES FOR P-GLYCOPROTEIN TRANSPORT IN VIVO

4.1. Introduction

Drug interactions constitute 6 to 15% of adverse event-related hospitalizations and are accountable for more than 100,000 deaths annually\(^1\),\(^2\). As combination chemotherapeutics become more prevalent, preclinical and translational studies to better predict and define drug interactions in patients are in increasing demand. Identification of membrane transporters through which these interactions may occur remains essential to predicting response and prevention. Common transporter proteins mediating clinical drug interactions exist within the SLC uptake superfamily and ABC efflux superfamily, including breast cancer resistance protein (BCRP), organic anion and cation transporters, and the most commonly evaluated P-glycoprotein (P-gp, MDR1, \textit{ABCB1})\(^3\). P-gp is an adenosine triphosphate (ATP)–dependent membrane glycoprotein responsible for extracellular efflux and plasma elimination of drugs. It is located in high concentrations in the brush border of epithelial membrane in kidney proximal tubules to facilitate removal from the bloodstream to urine, in the canalicular apical domain of hepatocytes to mediate against re-uptake from bile, in the apical brush border membrane of the small intestine to prevent absorption, and in the luminal side of the blood-brain barrier to
prevent penetration to the brain. Interactions occurring through this receptor have been responsible for up to 3 fold increase in typical plasma concentrations of substrates although even small increases present a vital concern for compounds portraying a narrow therapeutic window.

Lenalidomide and pomalidomide are novel thalidomide derivatives with anti-angiogenic, anti-neoplastic and immunomodulatory activities. Lenalidomide has been Food and Drug Association approved for multiple myeloma and anemia associated with myelodysplastic syndrome. Multiple myeloma therapeutics are commonly administered as polypharmacy. A recent manuscript unexpectedly observed a significant drug interaction of lenalidomide in combination with another chemotherapeutic and provides novel evidence for substrate interaction occurring through P-gp transport. In this clinical trial involving concomitant CCI-779 (Temsirolimus) and lenalidomide administration in the treatment of relapsed multiple myeloma, both compounds have altered PK profiles in the presence of combined therapy. Given concurrently, renal clearance of both drugs decreased significantly by nearly 2 fold. Pharmacokinetic parameters reveal decreased Cmax and increased AUC for fixed doses of lenalidomide corresponding with an increase in dose of CCI-779, and vice versa. Scrutiny of the literature suggested that this interaction may occur through P-gp. As CCI-779 is a known P-gp substrate and lenalidomide clearance occurs mainly through renal elimination with a suggested active elimination component, these findings led to the conduct of translational in vitro experiments to investigate the interaction through P-gp transporters.
Decreased intracellular concentrations of lenalidomide in Pgp-overexpressing cell lines were returned to baseline by concomitant incubation with CCI-779\textsuperscript{13}. This report confirmed \textit{in vitro} transport and drug interactions with lenalidomide can occur through P-gp, and suggest significant \textit{in vivo} transport may be the foundation for interactions observed with CCI-779.

This report generated public controversy in a corresponding letter to the editor as returned comments stated disbelief to the significant role of P-gp in the \textit{in vivo} absorption and disposition of lenalidomide\textsuperscript{18}. Celgene has separately identified lenalidomide as weak substrate of P-gp\textsuperscript{19}. To evaluate the potential for human DDIs as set forth by FDA guidelines and suggestions from the international transporter consortium, a clinical evaluation was performed in combination with digoxin, a known substrate of P-gp used as the current standard for assessment of clinical MDRI interactions\textsuperscript{20}. Although the combination resulted in 14\% increase in digoxin Cmax and 8\% increase in AUC, in their study this interaction fell below significant FDA guidelines\textsuperscript{21} to cause concern of future interactions.

In opposition to results reported from the lenalidomide-digoxin study, the clinical pharmacokinetic parameters of lenalidomide are very highly suggestive of active elimination mechanisms. Celgene itself has stated many times the likelihood of active elimination mechanisms. These claims are supported by evaluation of lenalidomide clearance in combination with physiological renal elimination mechanisms and rates.
90% of lenalidomide is excreted in urine\textsuperscript{17}, where on average of 42% can be cleared by glomerular filtration (the average glomerular filtration rate provided by the National Kidney Foundation is 110ml/min / lenalidomide clearance rate of 261mL/min\textsuperscript{17}). Therefore, in reference to data published by Celgene, approximately 50% of lenalidomide clearance may be due to active tubular secretion. Other transporters responsible for lenalidomide absorption or disposition have not been reported. Additionally, large differences in translational lenalidomide transport studies confirm that a significant P-gp interaction occurs in combination with CCI-779 which would likely be reflected \textit{in vivo}. Combined observances and \textit{in vitro} data strongly suggest that the interaction observed in clinic was mediated by interactions through P-gp. Thus, further evaluation is warranted to determine the significance of P-gp and other active transporters to lenalidomide absorption and disposition.

To substantiate the impact of P-gp on the \textit{in vivo} kinetics of the leading IMiD agents, we performed a comparative pharmacokinetic analysis in MDR1 knockout and FVB wild type mice. While humans have one gene encoding P-gp (ABCB1), mice have both mdr1a (mdr3) and mdr1b (mdr1) genes\textsuperscript{22, 23}. Studies in mdr1 and mdr3 gene knockout models in mice suggest the mdr3 (mdr1a) gene is responsible for P-gp expression and drug excretion in the renal proximal tubules and glomerular mesangium\textsuperscript{24-27}. The mdr1a/b knockout mouse model has previously been used to explore drug disposition dependence on P-gp, and have repeatedly shown enhanced bioavailability and reduced clearance of many known P-gp substrates and inhibitors\textsuperscript{28-32}. These mouse knockout models provide
convincing proof of the significance of P-gp transport to absorption, distribution and elimination on lenalidomide and pomalidomide. Comparative IV PK in P-gp knockout and wild type mice are aimed at characterizing the contributions from Pgp-altered clearance and distribution only. Oral IMiD administration in these separate colonies will identify Pgp-induced GI effects additional to altered clearance observed in IV mice, and will represent the net result which may be reflected in patients. Understanding of these agents as transporter substrates is important for prediction and prevention of unintentional drug-drug interactions with combined chemotherapeutics or common, non-chemotherapeutic medications.

4.2. Materials and Methods

4.2.1. Chemicals and Solvents
Pomalidomide was synthesized in the lab of Dr. Ching Shih Chen at The Ohio State University. Lenalidomide powder was purified from commercial capsules. The structure and purity of stock powders were confirmed by nuclear magnetic resonance and liquid chromatography mass spectrometry. Daily dosing solutions of 0.1mg/mL lenalidomide were prepared in PBS followed by sterile filtration. Daily stock solutions of pomalidomide were created as 1mg/ml in sterile DMSO followed by dilution into sterile PBS to reach a final dosing solution concentration of 0.1mg/mL. Genistein was HPLC grade (>98%) and was purchased from Sigma.
4.2.2. Mice

MDR1a/b knock out mouse breeding pairs were purchased from Taconic. The mouse colony used for the study was bred in accordance with agreements contracted through Taconic. Male and female mice were utilized in studies between 6 to 11 weeks of age. Age matched FVB wild type mice (male and female) were obtained from Harlan and acclimated to the facility for 48 hours before study initiation. Mice were given ad libitum access to soft food diets and placed on wire flooring cage inserts without bedding for the 3 days previous to the pharmacokinetic dose. Food supply consisted of Dietgel 76A on days 1-2, and RecoveryGel on day 3. Food was removed 30 minutes before the dark cycle on the third night and withheld until 5 hours post PK dosing on day 4. The study design was approved and performed in compliance with Institutional Animal Care and Use Committee guidelines.

4.2.3. Dosing and Pharmacokinetics

Prior to dosing, mice were assigned to groups to ensure uniform representation of age and sex amongst time points. Lenalidomide was dose-normalized to body weight with a dosing volume of 5ul per g mouse. Animals received drug by rapid bolus tail vein injections or oral gavage. Blood was collected from sacrificed mice by cardiac puncture at 5, 10, 20, 30, 45, 60, 90, 150, 240 and 360 minutes post dose for mice receiving lenalidomide, and additionally at 540 minutes in mice receiving pomalidomide. Plasma
was harvested immediately from heparinized tubes, placed directly on dry ice and stored at -80°C.

4.2.4. Sample Processing

Samples were thawed at room temperature and briefly vortex mixed. 100μL plasma aliquots were taken for processing and quantification. For lenalidomide samples, drug was extracted from plasma via liquid-liquid extraction using cold acetonitrile containing 200nM genistein as an internal standard. Supernatant was removed and evaporated in a rotary speed vacuum for 6 hours. The residue was reconstituted in HPLC grade H2O containing 0.1% Formic Acid and transferred to MS vials. For pomalidomide samples, 10μl of 10μg/ml hesperetin (internal standard) was spiked into 100μL plasma, and deproteinized and extracted using 1mL cold acetonitrile. Supernatant was removed and evaporated by nitrogen stream, and reconstituted in 120μL of 5% acetonitrile. For preparation of standard curves, lenalidomide and pomalidomide at 0.3 to 30,000nM were individually spiked into pooled mouse plasma (Valley Biomedical and Lampire Biological Laboratories).

4.2.5. LC/MS/MS

Quantification of lenalidomide was achieved using the high performance liquid chromatography tandem mass spectrometry (LC/MS/MS) assay described previously (Chapter 1). A similar assay was created and validated for quantification of pomalidomide. For both IMiD compounds, a flow gradient of water and ACN containing
0.1% formic acid was passed through a Zorbax C-18 extended column. Compounds were detected on a triple quadrupole mass spectrometer with an atmospheric pressure chemical ionization interface.

4.2.6. Pharmacokinetic Analysis

PK parameter estimates were generated from plasma using WinNonlin Professional version 5.2.1 (Pharsight Corporation). Noncompartmental analysis was performed using a linear up/log down regression analysis and a uniform weighting scheme. All reported parameters, including total body clearance (CL) and volume of distribution (Vz), Area under the concentration-time curve from zero to infinity (AUC), maximum achieved concentration (Cmax), and time to maximum concentration (Tmax), were estimated by non-compartmental analysis. Bioavailability was calculated as 

\[(\text{Dose}_{\text{Oral}} \times \text{AUC}_{\text{IV}}) / (\text{Dose}_{\text{IV}} \times \text{AUC}_{\text{Oral}}) \times 100.\]

4.3. Results

4.3.1. Oral lenalidomide

Lenalidomide at 0.5mg normalized to body weight was administered as an oral bolus dose in two groups of mice; wild type FVB mice (WT) with functional MDR1 isoforms and heterozygous mdr1a/mdr1b double knockout mice (here forth referred to as knockout; KO). Mice between the ages of 6 to 11 weeks were gender-matched and age-
matched by time point to facilitate direct comparison between WT and KO. Plasma was collected 5 to 280 minutes post gavage from 5 mice per time evaluation (n=50 per group; 100 mice total). Plasma concentration-time plots are displayed in figure 4.1. Maximum concentrations (Cmax) of 253 μg/mL in WT and 499 μg/mL in KO are achieved (Table 4.1). Lenalidomide plasma exposure in WT mice is 15.6 min*μg/mL, while an AUC of 24.0 min*μg/mL is estimated in the KO group. Mutation of functional P-gp does not alter the time to maximum concentration (Tmax).

Concentrations at the Tmax (10min) increase two fold in KO compared to WT (Figure 4.2) and are consistently elevated in KO following 30min. These combined effects shift the plasma half life from 39min in WT to 58min in KO; a 50% fold increase in mice without functional P-gp. Observed clearance (Cl) and volume of distribution (Vz) are reported without adjustment to bioavailability (F). No difference between groups is observed in the non-adjusted Vz parameters. However, CL/F of lenalidomide from the plasma compartment is decreased 50% in the KO group. Overall, increased plasma concentrations account for a 54% increase in the plasma exposure of orally administered lenalidomide in KO mice.

4.3.2. Intravenous lenalidomide

An identical dose to the previous study was administered intravenously to a new population of age-matched WT and KO mice. Selection of evaluation times and number of mice per evaluation was consistent with oral dosing groups. The initial evaluations at 5
minutes represent ‘peak’ concentrations, reaching an average of 635 μg/mL in the KO mice and 436 μg/mL in WT (Table 4.2 and figure 4.3). All subsequent evaluations demonstrate elevated plasma concentrations of lenalidomide in the KO group. Comparative analysis performed on pharmacokinetic parameters is shown in figure 4.4. In the group with nonfunctional P-gp, lenalidomide clearance is only 80% the efficiency observed in WT. Similar to lenalidomide administered orally, half life in KO was 44% increased when P-gp function was absent. Estimated plasma exposure in the WT mice was 19.3 min*μg/mL, while plasma exposure was increased 25% in the absence of P-gp.

AUC data from these four groups can be extrapolated to determine bioavailability. In the wild type mice, non-compartmentally estimated oral bioavailability is 80.8%. In the knockout mice, bioavailability is observed at near to 100%.

4.3.3. Intravenous pomalidomide
Pomalidomide has shown promising clinical results in multiple modalities and pre-clinical data support its potential for expanded application. This agent is currently administered in combination regimens and further combined clinical investigations are anticipated. To predict whether there is concern for a drug interaction to occur through P-gp, we apply the knockout model to examine the impact of P-gp on pomalidomide pharmacokinetics. 0.5mg/kg intravenous pomalidomide was administered to WT and KO mouse populations (55 mice per group; 110 mice total) employing the experimental
design described above with lenalidomide. The 5 minute average ‘peak’ concentrations are 3 times increased in the KO mice, reaching 886 μM. Overall the pomalidomide profile is greater than 3 fold increased at each evaluation apart from a pronounced ‘dip’ around 30 minutes rebounding to a peak at approximately 1.5 hours (Figure 4.5).

Pharmacokinetic analyses evaluate the clearance and volume of distribution at 6.6 mL/min/kg and 1.1 L/kg in KO and 18.3 mL/min/kg and 2.2 L/kg in WT, respectively (Table 4.3). Calculated fold change of KO/WT parameters suggests clearance is decreased 64% and Vz decreased by half their respective values in WT mice (Figure 4.6). The half-life of pomalidomide is increased approximately 40% and overall plasma exposure increased 2.7 fold in P-gp defective mice.

4.3.4. Oral pomalidomide

Comparative oral administration of 0.5mg/kg pomalidomide in these two groups of mice was intended to assess the additional role of P-gp to absorption. Again, gender- and age-matched mice of P-gp WT and KO genotypes were assigned to groups of 5 to quantify pomalidomide in the plasma 5 minutes to 540 minutes post dose. Initial plasma concentrations are lower in KO and gradually achieve an average 232 μM peak concentration at 45 minutes (Figure 4.7 and Table 4.4). In the WT group a Cmax of 313 μM is observed at 10 minutes. Although observed Tmax is different, in both groups concentrations vary only slightly between 5 and 45 minutes. Similar to the KO group in
the IV administration route, the KO oral PK profile shows a rapid decrease in concentration at 1 hour, followed by a second peak with a 4hr Tmax.

A 1.13 fold increase in the KO Vz/F parameter is observed upon comparative analysis (Figure 4.8). Half life in this group is increased 50% compared to WT. As observed in the WT mice, depletion of P-gp transport decreases pomalidomide clearance from plasma. AUC is effected 1.25 fold, increasing total plasma exposure in the KO mouse group. Oral bioavailability of pomalidomide in WT mice is estimated to be 114% and in KO estimated at 54%.

4.4. Discussion

Few clinical studies have directly evaluated the potential for lenalidomide drug-drug interactions, due primarily to its assumed straightforward pharmacokinetics. Among those that have directly evaluated pharmacokinetics include a small cohort of patients treated with digoxin\textsuperscript{20} and a second study looking at dexamethasone and lenalidomide\textsuperscript{33}. While both studies suggested the occurrence of drug-drug interactions, the magnitude of these effects were relatively small and within the FDA “no effect” boundaries\textsuperscript{21, 34}. However, these studies included only healthy volunteers and the n=6 cohorts used to make these assessments may not be representative of the broader population or the population of cancer patients who are the primary recipients of lenalidomide therapy.
These observations have been used to facilitate decisions regarding the safety of lenalidomide use and implications guiding drug combination trials. However, a recent clinical report suggested that this conclusion may have been underestimating the effect of P-gp to lenalidomide pharmacokinetics. Further in vitro analysis supported this hypothesis, as a significant interaction responsible for changes in lenalidomide flux and intracellular accumulation was mediated by P-gp. To readdress this concern in an in vivo setting, the experiments herein using P-gp knockout mice were performed.

Administration of lenalidomide and pomalidomide in wild type mice versus those lacking MDR1 genes confirm interaction of the leading IMiDs with the P-gp transporter. Without functional P-gp, we observe an increase in plasma exposure (AUC) of lenalidomide and pomalidomide in both IV and PO dosing routes. Elimination of functional P-gp decreases clearance of these agents in IV and PO administration and is supported by loss of P-gp in the renal tubules. Increased lenalidomide absorption in the GI tract is observed in the KO mice as calculated by oral bioavailability and is again supported by loss of P-gp in the apical brush border membrane of the GI tract. However, non-parametric results compared between oral and IV administrations of pomalidomide are complicated to interpret. Intravenous administration in the KO versus WT mice results in 2.7 fold increase in AUC, while oral administration results in only 1.27 fold increase in KO compared to WT mice. Therefore, the oral bioavailability calculated for pomalidomide in KO mice is 58%.
The pharmacokinetic profile for pomalidomide in mice suggests that mechanisms aside from P-gp mediate plasma exposure. Both IV and oral administration routes reveal a plasma ‘peak’ occurring 1.5 hours (IV) to 4 hours (oral) post dose in the KO and 30 minutes post dose in the WT regardless of administration route. Although these discrepancies may be overlooked as variability, these profile characteristics in mice have been reported previously, with a secondary ‘peak’ observed 5 hours post 10mg/kg IP injection in C57BL/6 mice36. The rationale behind this observation has yet to be determined. However, we hypothesize this secondary ‘peak’ to be characteristic of enterohepatic recirculation. Exploration of mass balance of IV pomalidomide in future studies would be useful to evaluate this potential.

Nonlinearities observed in previous mouse and human pharmacokinetics may be partially explained by influence from P-gp. In chapter three, we observed nonlinearity in plasma PK performed in mice given increasing doses of lenalidomide via IV bolus administration. We hypothesized this nonlinearity in part might be reflective of saturation of an active elimination mechanism. The confirmation of P-gp transporter dependence in the study herein provides an active renal transporter which may be responsible for this nonlinearity of lenalidomide. Additionally, lenalidomide was not detected in the brain at 0.5 and 1.5mg/kg doses in the mouse, but were quantifiable in every other tested tissue. P-gp transport of lenalidomide out of the brain at lower concentrations may become saturated at higher doses, accounting for immeasurable concentrations up to 1.5mg/kg, and low concentrations following higher doses of 5 and 10mg/kg. Although subtle, the
nonlinearities reported in mouse PK study are reflected in reports from clinical trials, where higher concentrations seem to follow a trend of decreasing clearance which is suggestive of active transporter saturation. Previous information discussed here provides evidence supporting that renal elimination via P-gp may be one such mechanism responsible for this phenomenon in humans.

P-gp is overexpressed in many malignant cell types and is enhanced moreso in primary multiple myeloma, chronic lymphocytic leukemia, and myelodysplastic syndrome cells from lenalidomide-treated and refractory patients. Although blocking systemic P-gp transport through unintentional drug-drug interactions may result in higher plasma concentrations and increase toxicity, there is also a positive potential for intentional drug interactions to have synergistic effects on MDR cells by enhancing intracellular concentrations. Studies of this nature have been underway to identify a compound able to overcome P-gp mediated MDR, although many compounds fail due to increased toxicity, nonspecific, and increased metabolism of these agents.

These studies provide significant implications for clinical use. Oral data provided herein would best reflect PK profile changes which may occur in patients, as both lenalidomide and pomalidomide are administered orally. These mouse experiments in oral administration take into account the net effect which may be observed in patients in ‘worst case’ scenario; i.e. 100% inhibited or defective P-gp. The FDA provides guidelines to assess a significance of transporters on disposition of evaluable substrates,
and is applied to delineate concern for potential clinical interactions\textsuperscript{21, 34}. Applying these criteria to this mouse data, lenalidomide PK is significantly affected by P-gp and is subject to interactions through this transporter. Pomalidomide data also shows significant alterations can result through changes in P-gp when given orally.

Mouse data of this nature can not be directly extrapolated to humans, but does provide insight and serves to predict and prevent serious drug interactions from occurring. Although the previous clinical investigation using digoxin predicts lenalidomide interactions through P-gp are not significant to affect pharmacokinetics\textsuperscript{20}, other clinical observations\textsuperscript{35} and the mouse data herein suggest otherwise. When giving concomitant administration of lenalidomide with known P-gp substrates and/or inhibitors, we suggest close monitoring of patients for unintentional drug interactions and increased adverse effects. Additionally, patients with single nucleotide polymorphisms (SNPs) in the ABCB1 transcript, low MDR protein expression, or defective P-gp function may be at risk for increased adverse events due to a significantly decreased clearance of lenalidomide. As lenalidomide has a narrow therapeutic window in certain malignancies\textsuperscript{41}, this caution is applied moreso when lenalidomide is used in these implications.

4.5. References


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**Figure 4.1.** Oral administration of lenalidomide in WT and KO mice.

Plasma concentrations measured 5 - 360 minutes post 0.5mg/kg lenalidomide via oral gavage. Error bars represent standard deviation from n=5 mice (n=50 per group).
Table 4.1. Non-compartmental parameter estimates from lenalidomide dosed orally. WinNonlin non-compartmental output from 0.5mg/kg lenalidomide oral gavage in WT and KO groups. Parameter estimates are calculated using a uniform weighting scheme and linear up/log down regression analysis. AUC, area under the estimated concentration-time curve; Vz_F, volume of distribution/bioavailability; Cl_F, clearance/bioavailability; HL_Lambda_z, half-life for each phase; Tmax, time to maximum concentration; Cmax, maximum estimated concentration.

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Figure 4.2. Fold change in PK parameter estimates: Oral lenalidomide.

Fold change calculated as KO/WT for each parameter (non-compartmental).
**Figure 4.3.** IV administration of lenalidomide in WT and KO mice.

Plasma concentration-time plot for 0.5mg/kg lenalidomide via IV bolus tail vein injection. Concentrations measured 5-360 minutes post dose. Error bars represent standard deviation from n=5 mice (n=50 per group).
### Table 4.2. Non-compartmental parameter estimates from IV lenalidomide.

WinNonlin non-compartmental output from 0.5mg/kg lenalidomide IV in WT and KO groups. Parameter estimates are calculated using a uniform weighting scheme and linear up/log down regression analysis. AUC, area under the estimated concentration-time curve; Vz, volume of distribution; Cl, clearance; HL_Lambda_z, half-life for each phase; Tmax, time to maximum concentration; Cmax, maximum estimated concentration.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type</th>
<th>P-gp KO</th>
<th>KO/WT</th>
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<tbody>
<tr>
<td>AUCall min*μg/mL</td>
<td>19.29</td>
<td>24.11</td>
<td>1.25</td>
</tr>
<tr>
<td>Vz_obs L/kg</td>
<td>1.32</td>
<td>1.52</td>
<td>1.15</td>
</tr>
<tr>
<td>Cl_obs mL/min/kg</td>
<td>25.67</td>
<td>20.53</td>
<td>0.80</td>
</tr>
<tr>
<td>HL_Lambda_z min</td>
<td>35.72</td>
<td>51.35</td>
<td>1.44</td>
</tr>
<tr>
<td>Tmax min</td>
<td>5</td>
<td>5</td>
<td>1.00</td>
</tr>
<tr>
<td>Cmax ng/mL</td>
<td>635</td>
<td>466</td>
<td>0.73</td>
</tr>
</tbody>
</table>

**IV Injection**

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>P-gp KO</th>
<th>KO/WT</th>
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</thead>
<tbody>
<tr>
<td>AUCall min*μg/mL</td>
<td>19.29</td>
<td>24.11</td>
<td>1.25</td>
</tr>
<tr>
<td>Vz_obs L/kg</td>
<td>1.32</td>
<td>1.52</td>
<td>1.15</td>
</tr>
<tr>
<td>Cl_obs mL/min/kg</td>
<td>25.67</td>
<td>20.53</td>
<td>0.80</td>
</tr>
<tr>
<td>HL_Lambda_z min</td>
<td>35.72</td>
<td>51.35</td>
<td>1.44</td>
</tr>
<tr>
<td>Tmax min</td>
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<tr>
<td>Cmax ng/mL</td>
<td>635</td>
<td>466</td>
<td>0.73</td>
</tr>
</tbody>
</table>
Figure 4.4. Fold change in PK parameter estimates: IV lenalidomide.

Fold change calculated as KO/WT for each parameter (non-compartmental).
Figure 4.5. IV administration of pomalidomide in WT and KO mice.

Plasma concentrations measured 5 - 540 minutes post 0.5mg/kg pomalidomide via IV bolus tail vein injection. Error bars represent standard deviation from n=5 mice (n=55 per group).
### Table 4.3. Non-compartmental parameter estimates from IV pomalidomide.

WinNonlin non-compartmental output from 0.5mg/kg pomalidomide IV in WT and KO groups. Parameter estimates are calculated using a uniform weighting scheme and linear up/log down regression analysis. AUC, area under the estimated concentration-time curve; Vz, volume of distribution; Cl, clearance; HL\_Lambda\_z, half-life for each phase; Tmax, time to maximum concentration; Cmax, maximum estimated concentration.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type</th>
<th>P-gp KO</th>
<th>KO/WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUCall min*μg/mL</td>
<td>26.92</td>
<td>72.99</td>
<td>2.71</td>
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<tr>
<td>Vz_obs L/kg</td>
<td>2.16</td>
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<tr>
<td>Cl_obs mL/min/kg</td>
<td>18.30</td>
<td>6.57</td>
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<tr>
<td>HL_Lambda_z min</td>
<td>81.71</td>
<td>113.62</td>
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<td>Tmax min</td>
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<tr>
<td>Cmax ng/mL</td>
<td>292</td>
<td>886</td>
<td>3.04</td>
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</table>
Figure 4.6. Fold change in PK parameter estimates: Pomalidomide IV.

Fold change calculated as KO/WT for each parameter (non-compartmental).
Figure 4.7. Oral administration of pomalidomide in WT and KO mice.

Plasma concentrations measured 5 - 540 minutes post 0.5mg/kg pomalidomide via oral gavage. Error bars represent standard deviation from n=5 mice (n=55 per group).
Table 4.4. Non-compartmental parameter estimates from PO pomalidomide.

WinNonlin non-compartmental output from 0.5mg/kg pomalidomide oral gavage in WT and KO groups. Parameter estimates are calculated using a uniform weighting scheme and linear up/log down regression analysis. AUC, area under the estimated concentration-time curve; Vz_F, volume of distribution/bioavailability; Cl_F, clearance/bioavailability; HL_Lambda_z, half-life for each phase; Tmax, time to maximum concentration; Cmax, maximum estimated concentration.

<table>
<thead>
<tr>
<th>Oral Gavage</th>
<th>Wild Type</th>
<th>P-gp KO</th>
<th>KO/WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUCall (min*μg/mL)</td>
<td>30.60</td>
<td>39.15</td>
<td>1.28</td>
</tr>
<tr>
<td>Vz_F_obs (L/kg)</td>
<td>2.16</td>
<td>2.44</td>
<td>1.13</td>
</tr>
<tr>
<td>Cl_F_obs (mL/min/kg)</td>
<td>15.97</td>
<td>12.12</td>
<td>0.76</td>
</tr>
<tr>
<td>HL_Lambda_z (min)</td>
<td>93.66</td>
<td>139.35</td>
<td>1.49</td>
</tr>
<tr>
<td>Tmax (min)</td>
<td>10</td>
<td>45</td>
<td>4.50</td>
</tr>
<tr>
<td>Cmax (ng/mL)</td>
<td>313</td>
<td>232</td>
<td>0.74</td>
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<tr>
<td>Oral Bioavailability</td>
<td>113.68</td>
<td>53.64</td>
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</table>
Figure 4.8. Fold change in PK parameter estimates: Oral pomalidomide.

Fold change calculated as KO/WT for each parameter (non-compartmental).
5.1. Conclusions

The leading immunomodulatory drugs lenalidomide and pomalidomide have proven efficacy and shown great promise in treating a vast population of diseases\(^1\text{--}^\text{15}\). It is likely that further pre-clinical testing may continue to reveal new therapeutic uses for these agents. However, the exact mechanistic targets of IMiDs have yet to be determined. Mouse pharmacokinetic studies needed to corroborate these efforts have not previously been published. Additionally, recent data has shown promise for lenalidomide to be a substrate for the efflux transporter P-glycoprotein (P-gp). As P-gp mediates transport of other therapies coadministered with these IMiDs, there is a potential for drug-drug interactions to occur. Given the life-threatening adverse reactions that occur with lenalidomide or pomalidomide single therapy\(^\text{16}\), these effects may be augmented by drug-drug interactions and lead to drug related mortalities. Therefore the potential for interactions occurring through P-gp should be thoroughly investigated for both agents.

Studies described herein have been aimed to address these concerns. To carry out these investigations, our group first created and validated a method for sensitive detection and quantification of lenalidomide in plasma and tissues of mice. The LC/MS-MS method
provides linear quantification of drug from concentrations 0.3nM to 30uM in 100uL mouse plasma with acceptable between batch variability. The assay was also applied to 10 other tissues collected from mice and was shown to provide good linearity and specificity in all tissues and acceptable recovery and overall process efficiency in all tissues except intestine. Pilot studies in mice confirmed the repeatability of this assay when applied to plasma and tissues of dosed mice.

Our group applied this method to characterize plasma pharmacokinetics given multiple intravenous, intraperitoneal and orally gavaged doses. In pharmacokinetic experiments described herein, we compared mouse plasma exposures to those reported on average in patients given a 25mg oral capsules and concluded equivalency of an IV dose of 1.5mg/kg in mice. We also report that optional intraperitoneal and oral dosing routes result in acceptable bioavailability in mice and provide good alternative dosing routes for multi-day dosing. A maximum tolerated dose was not achieved due to limited solubility of lenalidomide in the chosen dosing vehicle.

We also characterized disposition of lenalidomide in mice dosed intravenously through quantification in plasma and tissues over time. With increasing doses from 0.5 to 10mg/kg, lenalidomide exhibits nonlinear kinetics in the plasma of mice. This nonlinearity is further perceived in tissues where distribution ratios show the lowest percentage of lenalidomide distribution resulting from a 1.5mg/kg dose. Accumulation is reported in spleen at all doses and in muscle at 10mg/kg dose. Lenalidomide distribution
into brain was not detected at lower administered doses but is quantified at low concentrations following doses of 5mg/kg and above.

Lenalidomide’s interaction with P-gp transporters *in vitro* has recently been confirmed\textsuperscript{17}. This data is suggestive that the clinical interaction observed between lenalidomide and a known P-gp substrate, temsirolimus, was the result of a P-gp induced drug-drug interaction. These results have significant implications due to potential interaction with other medications and were subject to question by the Celgene scientists, the company that markets lenalidomide. We therefore sought to definitively confirm that lenalidomide interacts with P-gp and plays a significant role in plasma exposure using a genetic approach *in vivo*. We performed comparative pharmacokinetic evaluations in mice with and without functional P-gp. At a dose of 0.5mg/kg, systemic exposure following orally administered lenalidomide increased 54\% in mice lacking P-gp as compared to wild type mice with functional protein, and following IV administration increased 25\% in the knockout mice. Oral bioavailability is calculated at 81\% in wild type mice, and 99\% in MDR1a/b knockout mice. Together these data establish that both renal and intestinal P-gp are responsible for disposition of lenalidomide.

Pomalidomide was additionally evaluated as a substrate for P-gp *in vivo* using the P-gp knockout mouse and experimental design described above. Following intravenous administration at 0.5mg/kg, pomalidomide systemic concentrations were 2.7 fold increased with an approximate 40\% increase in half life with the loss of P-gp. These same
parameters following oral administration were 28% and 50% increased, respectively, in P-gp knockout mice compared to wild type. Our data conclude that pomalidomide is also a P-gp substrate \textit{in vivo}. These studies demonstrate with certainty that lenalidomide and pomalidomide are MDR1 substrates and have potential clinical implications given the narrow therapeutic index of these agents in several diseases.

5.2. Future Perspectives and Significance

The leading IMiDs are very highly utilized drugs with approved and prospective clinical applications spanning a broad range of malignancies. Lenalidomide is approved in MM\textsuperscript{1} and MDS\textsuperscript{2} and has been scrutinized in more than 400 clinical investigations since the drug’s release 10 years ago (clinicaltrials.gov). Initial clinical investigations of pomalidomide are more recent, albeit showing similar activity profiles with the potential for greater potency than lenalidomide\textsuperscript{14, 15} and the ability to treat lenalidomide-refractory patients\textsuperscript{18}. The National Cancer society predicts 30,000 to 40,000 individuals living with MDS and a prevalence of 45,000 to 50,000 people with MM. In addition to the number of patients with other malignancies where both IMiDs show great potential, the long-term impact of the work described herein may affect the lives of millions of individuals worldwide.
Pre-clinical and translational therapeutic efficacy and toxicities of these agents have been studied in mice without regard to pharmacokinetic data to guide selection of doses resembling those in humans. Our data suggests that previous lenalidomide doses administered to mice at 50mg/kg$^{19-21}$ and 100mg/kg$^{22}$ far exceed therapeutic plasma exposures achieved in patients$^{1,23-25}$, and thus the translational equivalency of this murine data is questionable. Using equivalent doses calculated herein, we have evaluated efficacy of lenalidomide in the Tcl-1 mouse model of CLL and in immune deficient SCID/NK depleted TCL-1 allograft mice. Daily doses of 0.5 and 5mg/kg IP had no significant effect on survival, disease progression, white blood cell counts or B-cell activation markers (data not shown). As it is likely for pre-clinical and translational murine studies to continue with this compound, our PK data reported herein should help guide the selection of these doses in future studies. Our conclusion that lenalidomide exhibits non-linear kinetics in mice is additionally of interest in choosing doses for efficacy and toxicity studies as we show that doubling the administered dose will not likely result in doubling the plasma (or target site) drug exposures.

Our characterization of lenalidomide tissue disposition can assist in predicting efficacy or supporting mechanisms in diseases which target the central nervous system or lymphoid organs. We show that concentrations of lenalidomide are low in the brain, and that tissue pharmacokinetic profiles exhibit additional nonlinearities and display aberrant patterns in muscle and spleen. The presence of an absorption phase and short concentration plateau in collected spleen and muscle tissue data indicates accumulation in these tissues which
may correlate with increased efficacy or toxicity of lenalidomide in diseases such as CLL which target lymphoid organs. Low concentration of lenalidomide in the brain supports the seldom observation of clinical neurotoxicity\(^{26, 27}\) and suggests limited efficacy in treating tumors of the central nervous system.

The aberrant PK patterns in muscle, spleen and brain along with further nonlinearities observed in lenalidomide plasma and tissue kinetics are suggestive of transporter mediated disposition. The discovery of lenalidomide transport through P-gp\(^{17}\) aids in the explanation of these kinetics, including the low concentrations observed in brain, but does not account for all observed occurrences. The accumulation observed in spleen and muscle may occur from an increased affinity of lenalidomide specific to these tissues. Other transporters responsible for lenalidomide and pomalidomide mediated transport, absorption, and distribution have not yet been identified. The very rapid oral absorption of lenalidomide and AUC saturation pattern observed in plasma is characteristic and suggestive of an uptake transporter function. As lenalidomide and pomalidomide structural similarities to nucleosides such as uracil (figure 5.1), it is possible that active nucleoside transporters may also mediate IMiD absorption and disposition in both mice and humans.

We have identified that both lenalidomide and pomalidomide are substrates for p-glycoprotein in the mouse, and that plasma pharmacokinetics can be altered when the function of these proteins is eradicated. This finding is crucial in determining safety and
efficacy of these agents in clinical settings, as transporters which mediate drug absorption, distribution and elimination are commonly the culprits of drug-drug interactions\textsuperscript{28}. The estimated cost of drug related morbidity and mortality in the past decade is estimated to exceed $177.4 billion annually\textsuperscript{29}, with drug interactions constituting 6 to 15% of these and resulting in greater than 100,000 deaths annually\textsuperscript{30, 31}. Thus, strategies and pre-clinical data to predict and prevent drug related events are in critical demand. Our translational data using the MDR1a/b knockout mice has addressed this need within the leading IMiD agents. As a further aid in the characterization of P-gp mediated IMiD interactions, in vivo substrates digoxin and loperamide and inhibitors cyclosporine and quinidine can be applied in mouse models as an effective pre-clinical model to predict the impact of interacting therapeutic agents in humans\textsuperscript{28}. To define clinical significance relevant to drug interactions, the FDA declares ‘no effect’ bounds to fall within 80% - 125% change in plasma exposure (or 90% confidence interval). Applying these criteria to our murine P-gp knockout investigation, we report that the P-gp mediated effect on disposition and elimination of the IMiDs is, in fact, significant. Therefore, we identify a potential for aggressive combination regimens used to treat refractory or advanced diseases to result in unintentional increased toxicity or life threatening drug-drug interactions occurring through P-gp. With the emergence of lenalidomide and pomalidomide in combinatorial drug trials (clinical trials.gov), a complete understanding of additional drug-transporter interaction is critical to maximize efficacy and minimize toxicity due to drug-drug interactions. Studies of this nature should continue to be actively pursued within the IMiD agents.
It is estimated that half of all cancer related deaths result from the evolution of multi-drug resistant (MDR) tumor cells\textsuperscript{32}, thus making MDR mechanisms a popular combination-therapeutic target. The most common and thoroughly studied mechanism for chemotherapy resistance is overexpression of P-gp in the cancer cell membrane\textsuperscript{33}. This mechanism has been identified in chronic lymphocytic leukemia\textsuperscript{34}, multiple myeloma\textsuperscript{35} and the myelodysplastic syndromes\textsuperscript{36}, thus likely mediating resistance of the IMiD agents in these common indications. Many attempts have been made to reverse or inhibit P-gp mediated transport to overcome MDR and increase therapeutic drug concentrations to systemic, intracellular, and P-gp protected sites such as the brain. These inhibitory mechanisms include noncompetitive inhibition of P-gp transport\textsuperscript{37}, downregulation of P-gp expression\textsuperscript{38-40}, steric inhibition of substrate binding\textsuperscript{41}, and noncompetitive and competitive inhibition of P-gp ATPase\textsuperscript{42, 43}. Unfortunately, most attempts to overcome resistance by these mechanisms \textit{in vivo} have been unsuccessful due to compound metabolism and a lack of potency and specificity. Although the use of successful P-gp inhibitory agents may augment IMiD efficacy against both resistant, refractory diseases by inhibition of membrane efflux and central nervous system tumors by inhibition of efflux by the blood brain barrier, it is not currently clear if this will increase lenalidomide-induced neurotoxicity.

Information provided herein can be influential in guiding further pharmacokinetic, efficacy, and drug interaction investigations of IMiDs in mice. Conclusive evidence that
P-gp effects drug disposition and pharmacokinetics should caution clinicians regarding drug interactions which may result when combining IMiDs with substrates and inhibitors of P-gp. The widespread modality, prevalence and expansive use of these agents are evident to the long-term impact of this work.

5.3. References


lenalidomide and pomalidomide inhibit the proliferation and function of T regulatory cells, Cancer Immunol Immunother 2009, 58:1033-1045


38. Donmez Y, Akhmetova L, Iseri OD, Kars MD, Gunduz U: Effect of MDR modulators verapamil and promethazine on gene expression levels of MDR1 and MRP1 in doxorubicin-resistant MCF-7 cells, Cancer Chemother Pharmacol 2011, 67:823-828


A. Uracil

B. Lenalidomide

C. Pomalidomide

Figure 5.1. The structural similarities of lenalidomide, pomalidomide and uracil


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