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Kavya Annu

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Abstract

It is a well-established phenomenon that dietary components containing CYP3A inducers or inhibitors if co-administered with drugs that are CYP3A4 substrates lead to marked drug-drug interactions. Because vitamin D is known to regulate intestinal CYP3A expression and gut CYP3A expression plays an important role in pre-systemic metabolism of CYP3A drugs, we determined the impact of vitamin D (VD3) status on systemic exposure and efficacy of chemotherapeutic agents that are CYP3A substrates. We employed VD3 sufficient and deficient mice to perform pharmacokinetics (PK) and anti-leukemic efficacy studies.

First, using hCYP3A4 transgenic mouse model we evaluated the intestinal, hepatic and renal expression of hCYP3A4, mouse CYPs and transporters in VD3 sufficient vs. deficient mice. We observed that female VD3 deficient mice had significantly lower duodenal mouse Cyp3a11 and hCYP3A4 expression than VD3 sufficient mice. Since intestinal CYP3A plays a significant role in first pass metabolism of drugs that are administered orally, chemotherapeutic agents such as dexamethasone (DEX) and dasatinib which are given orally to the patients were chosen to study the effect of VD3 status on systemic exposure of these CYP3A4 substrates.

In C57BL/6 mice, we performed dexamethasone PK study, where we observed male VD3 deficient mice had higher plasma DEX levels compared to sufficient mice on day 3.5. No significant differences were observed in females.

Another CYP3A4 substrate, dasatinib PK was also performed where AUC's were not significantly different between groups. However, during early absorption at $t = 0.25$ hr, VD3 deficient male mice had significantly higher dasatinib plasma levels compared to VD3 sufficient mice. In conclusion, VD3 levels (sufficient vs. deficient) significantly altered intestinal Cyp3a expression in female hCYP3A4 mice, and in male's, plasma exposure was different for orally administered DEX at 3.5 days, dasatinib at 0.25 hr. Ultimately, there was no significant effect of VD3 status on DEX or dasatinib PK indicating no potential vitamin drug interactions. However, DEX being a potential CYP3A inducer, we also observed significant induction of multiple mouse Cyp3a's in intestine of VD3 deficient but not VD3 sufficient mice. This differential induction of Cyp3a's due to vitamin D status could lead to drug interactions with other co-administered CYP3A substrates.

Given the high prevalence of VD3 deficiency in acute lymphoblastic leukemia (ALL) patients, we evaluated the effect of vitamin D deficiency on survival outcome from pre-B cell Arf -/- BCR-ABL acute lymphoblastic leukemia in a murine model. Vitamin D sufficient mice died earlier (p

To investigate the mechanism by which vitamin D affected the survival and disease growth in VD3 sufficient mice, we performed in vitro studies and demonstrated 1, 25-dihydroxy vitamin D (1, 25-(OH)2VD3) increased the number of BCR-ABL ALL cells only when co-cultured with bone marrow stroma. 1, 25-(OH)2VD3 induced CXCL12 production in vivo and in vitro in bone marrow stromal cells and CXCL12 increased stromal migration and the number of BCR-ABL blasts. Vitamin D together with ALL reprogrammed the marrow environment by increasing production of Type I and V collagens, potentially trapping ALL blasts and CXCL12 expression to support tumor progression and leukemia cell homing.

In conclusion, although vitamin D deficiency did not cause significant interactions with anti-leukemic CYP3A4 substrates, there was a significant effect on disease progression and survival of mice from BCR-ABL ALL.

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UNIVERSITY OF TENNESSEE HEALTH SCIENCE CENTER

DOCTORAL DISSERTATION

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Acute Lymphoblastic Leukemia Mouse Model but
Do Not Cause Vitamin-Drug Interactions**

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The University of Tennessee*

in

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DEDICATION

To my beloved parents, husband, siblings and my friends.
I am and will forever be grateful for your unconditional love, patience and inspiration.

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With a number of people that made my journey not only effortless but also eventful and memorable, I would like to express my sincere gratitude to everyone that has been a part of my journey here at University of Tennessee Health Science Center and St. Jude Children's Research Hospital.

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ABSTRACT

It is a well-established phenomenon that dietary components containing CYP3A inducers or inhibitors if co-administered with drugs that are CYP3A4 substrates lead to marked drug-drug interactions. Because vitamin D is known to regulate intestinal CYP3A expression and gut CYP3A expression plays an important role in pre-systemic metabolism of CYP3A drugs, we determined the impact of vitamin D (VD₃) status on systemic exposure and efficacy of chemotherapeutic agents that are CYP3A substrates. We employed VD₃-sufficient and -deficient mice to perform pharmacokinetics (PK) and anti-leukemic efficacy studies.

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Given the high prevalence of VD₃ deficiency in acute lymphoblastic leukemia (ALL) patients, we evaluated the effect of vitamin D deficiency on survival outcome from pre-B cell *Arf*^{-/-} BCR-ABL acute lymphoblastic leukemia in a murine model. Vitamin D sufficient mice died earlier (p<0.003) compared to vitamin D deficient mice.

To investigate the mechanism by which vitamin D affected the survival and disease growth in VD₃ sufficient mice, we performed *in vitro* studies and demonstrated 1, 25-dihydroxy vitamin D (1, 25-(OH)₂VD₃) increased the number of BCR-ABL ALL cells

only when co-cultured with bone marrow stroma. 1, 25-(OH)₂VD₃ induced CXCL12 production *in vivo* and *in vitro* in bone marrow stromal cells and CXCL12 increased stromal migration and the number of BCR-ABL blasts. Vitamin D together with ALL reprogrammed the marrow environment by increasing production of Type I and V collagens, potentially trapping ALL blasts and CXCL12 expression to support tumor progression and leukemia cell homing.

In conclusion, although vitamin D deficiency did not cause significant interactions with anti-leukemic CYP3A4 substrates, there was a significant effect on disease progression and survival of mice from BCR-ABL ALL.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ABCB1	ABC subfamily B member 1
ABCC4	ABC subfamily C member 4
ABCG2	ABC subfamily G member 2
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
BCRP	Breast cancer resistant protein
BM	Bone marrow
BMD	Bone mineral density
BMM	Bone marrow microenvironment
CAR	Constitutive androstane receptor
CARs	CXCL12 abundant reticular cells
Cl	Clearance
CLL	Chronic lymphoid leukemia
CML	Chronic myeloid leukemia
CYP	Cytochrome P450
DBP	Vitamin D binding protein
DEX	Dexamethasone
GR	Glucocorticoid receptor
HAT	Histone acetylase activity
HBSS	Hank's balanced salt solution
hMSCs	Human marrow-derived stromal cells
HPMC	Hydroxypropyl methyl cellulose
HSCs	Hematopoietic stem cells
IL-2	Interleukin-2
MRP4	Multidrug resistance associated protein 4
MSCs	Mesenchymal stem cells
NHL	Non-Hodgkin's lymphoma
NR	Nuclear hormone receptor
NR1I1	NR subfamily 1, group 1, member 1
NR1I2	NR subfamily 1, group 1, member 2
NR1I3	NR subfamily 1, group 1, member 3
PGP	P-glycoprotein
Ph+	Philadelphia chromosome
PK	Pharmacokinetics
PTH	Parathyroid hormone
PXR	Pregnane X Receptor
RXR	Retinoid X Receptor
SDF-1	Stromal cell derived factor 1
SRC-1	Steroid receptor activator 1
SRC-2	Steroid receptor activator 2
SRC-3	Steroid receptor activator 3
V1	Volume of distribution

VD ₃	Vitamin D
VDD	Vitamin D deficiency
VDR	Vitamin D Receptor
VDREs	Vitamin D response elements
1, 25-(OH) ₂ VD ₃	1, 25-dihydroxy vitamin D
25-(OH) ₂ VD ₃	25-hydroxy vitamin D

CHAPTER 1. INTRODUCTION

Cytochrome P450s (CYP) play significant roles in detoxification of many exogenous chemicals, drugs and toxins. These enzymes have broad substrate specificity and metabolize various endogenous and natural products as well. CYP's are not only involved in drug disposition but also contribute significantly to first-pass metabolism of many drugs. Various drugs and endogenous compounds like vitamin D can transcriptionally induce CYP3A. Depending on the dose and route of inducing agents, the extent of intestinal and hepatic induction may differ. Moreover, intestinal CYP3A expression is sensitive to seasonal changes correlating with seasonal vitamin D levels (Thirumaran et al., 2012). Consequently the induction of CYP's can lead to decreased oral bioavailability of many drugs that are metabolized by CYP3A (Kaminsky & Fasco, 1991). The following chapter highlights the role of vitamin D in transcriptional regulation of cytochrome P450s through various receptors and associated drug interactions.

Overview of Vitamin D

Vitamin D Bioactivation

Vitamin D (VD_3) is a seco-steroid hormone which can be synthesized from 7-dehydrocholesterol when skin is exposed to ultraviolet radiation. VD_3 is hydroxylated in the liver into 25-hydroxy vitamin D (25-(OH)VD_3) by two different vitamin D-25-hydroxylases (R. Kumar, 1984): CYP27A1, CYP2R1 which are present in hepatic microsomes and mitochondria. 25-(OH)VD_3 is the major circulating form of VD_3 (Henry & Norman, 1984) and it tightly binds to vitamin D binding protein (DBP) in the plasma. It has a long plasma half-life (~14 days) and is generally used as a measure of vitamin D status (Holick, 2007). 25-(OH)VD_3 is further hydroxylated into a biologically active form, 1,25-dihydroxy vitamin D ($1,25\text{-(OH)}_2\text{VD}_3$) mainly by CYP27B1 in kidney (Hewison et al., 2007). However, CYP27B1 is also expressed in extra-renal tissues such as intestinal epithelial cells, keratinocytes, dendritic cells, cancer cells etc. where $1,25\text{-(OH)}_2\text{VD}_3$ is locally formed (Feldman, Krishnan, Swami, Giovannucci, & Feldman, 2014; Gao, Liao, Han, Thummel, & Mao, 2018). Well known intestinal effects mediated by $1,25\text{-(OH)}_2\text{VD}_3$ could be a result of local bioactivation of VD_3 via CYP27B1 (Gawlik et al., 2015). CYP27B1 not only increases $1,25\text{-(OH)}_2\text{VD}_3$ levels but also decreases its metabolite, $24,25\text{-(OH)}_2\text{VD}_3$ levels by converting it into $1,24,25\text{-(OH)}_3\text{VD}_3$ (Inouye & Sakaki, 2001).

Vitamin D Inactivation

Both 25-(OH)VD_3 and $1,25\text{-(OH)}_2\text{VD}_3$ are converted into polar metabolites via two specific pathways, the first step being the hydroxylation of $1,25\text{-(OH)}_2\text{VD}_3$ to $1,24\text{R},25\text{-(OH)}_3\text{VD}_3$ and further oxidation leading to a terminal product, calcitroic acid

(Endres & DeLuca, 2001). CYP24A1 (24-hydroxylase) is the enzyme that catalyzes the hydroxylation at C-24 and C-23 positions of vitamin D metabolites, which is physiologically the main route of vitamin D degradation (Christakos, Dhawan, Verstuyf, Verlinden, & Carmeliet, 2016). CYP24A1 is the mitochondrial P450 mainly expressed in kidney and other vitamin D target tissues such as intestine (R. Kumar, 1984) and bone (Turner et al., 1980). The other P450 enzyme which can also metabolize 1,25-(OH)₂VD₃ was later reported to be CYP3A4 contributing to form 23R- and 24S- hydroxy metabolites, which are stereo chemically opposite to the metabolites produced by CYP24A1 (Xu et al., 2006). Hydroxylation at C-23 is preferred by CYP3A4 and represents a dominant pathway of 1,25-(OH)₂VD₃ metabolism in liver and intestine.

Vitamin D Regulation

Target cells of vitamin D have the ability to regulate its metabolism at the cellular level indicating the importance of cellular concentration of 1,25(OH)₂VD₃ by which tissues regulate their responsiveness to vitamin D (G. Jones, Strugnell, & DeLuca, 1998). Moreover, vitamin D bio-activation and degradation is tightly regulated to maintain appropriate levels of active vitamin D and its physiological effects (G. Jones et al., 1998). For example, 1,25-(OH)₂VD₃ can itself stimulate CYP24A1 to increase its metabolism whereas low calcium and parathyroid hormone (PTH) levels can inhibit CYP24A1 to maintain the active vitamin D levels (Christakos et al., 2016). Similar to this negative feedback mechanism in kidney, CYP3A4 is also prone to negative feedback mechanism by constitutive 1,25-(OH)₂VD₃ in intestine (Xu et al., 2006). It has been reported that 1,25-(OH)₂VD₃ can regulate intestinal Cyp3a in mice (Makishima et al., 2002), suggesting that induction of CYP3A4 by 1,25(OH)₂VD₃ can in turn result in enhanced metabolism of 1,25(OH)₂VD₃ itself, thereby mitigating the extent of its cellular effects.

Vitamin D and Vitamin D Receptor

Different forms of vitamin D can bind to DBP which transports vitamin D between tissues such as liver, kidney, skin and to various target tissues including intestine and bone. When the biologically active form of vitamin D, 1,25-(OH)₂VD₃ enters plasma, it is taken up by target tissues expressing a cytosolic receptor known as the vitamin D receptor (VDR). 1,25(OH)₂VD₃ binds to VDR with high affinity, generally in the range of 10⁻¹⁰ M (DeLuca & Schnoes, 1976).

VDR (NR1I2) belongs to nuclear receptor (NR) superfamily and acts as a transcription factor when ligand activated. When 1,25-(OH)₂VD₃ binds to VDR, the complex forms a heterodimer with the retinoid X receptor (RXR) and binds to vitamin D response elements (VDREs) on target DNA sequence with high affinity. Following this, recruitment of transcriptional coactivators, such as steroid receptor activator 1, 2, 3 (SRC-1, SRC-2, SRC-3) that has histone acetylase activity (HAT), is required to either activate or repress transcription of target genes. These coactivators bind to the AF2

domain of the liganded VDR and act as master regulators of 1,25-(OH)₂VD₃ action (Christakos et al., 2016; Haussler et al., 2013).

VDR is expressed in many tissues *in vivo* but at relatively low levels compared to its main target tissues such as bone, kidney and intestine which have high VDR expression (Dame, Pierce, & DeLuca, 1985). It is also expressed in immune cells, specially T-cells where it modulates interleukin -2 (IL-2) levels (Dame et al., 1985).

Overview of Vitamin D

CYP3A4/Cyp3a Gene

CYP enzymes are a superfamily of hemeproteins that metabolize a wide range of xenobiotics including therapeutic drugs, toxins, carcinogens as well as endogenous molecules (Nebert & Russell, 2002). Three P450 families CYP1, CYP2, CYP3 are currently known to be involved in drug metabolism (Furge & Guengerich, 2006). In humans, the CYP3A subfamily is critical for drug clearance and is reported to metabolize at least 50% of the currently prescribed drugs (Y. T. Liu, Hao, Liu, Wang, & Xie, 2007) (Guengerich, 2008). The CYP enzymes not only play a significant role in therapeutic drug metabolism and disposition, but they are also responsible for many adverse drug reactions. Clinical data suggests sex and age dependent tissue distribution of CYP enzymes and huge interindividual variability.

Localization of CYP3A4/Cyp3a

Most CYP enzymes are highly expressed in tissues involved in absorption, metabolism and excretion such as liver, small intestine and kidney. Almost 70% of human P450's is composed of CYP1A2, 2A6, 2B6, 2C, 2D6, 2E1 and 3A isoforms. CYP3A4, CYP3A5, CYP3A7 and CYP3A43 are the four isoforms of the CYP3A family among which CYP3A4 and CYP3A5 account for almost 30% and 20% of total P450 respectively. In humans, CYP3A4 is highly expressed in mucosal epithelium of duodenum and jejunum (Paine et al., 1997) (Watkins et al., 1985). It is also predominantly expressed in normal liver (Achour, Barber, & Rostami-Hodjegan, 2014). Hepatic distribution of CYP's is relatively homogenous compared to small intestine where both the content and activity of CYPs is higher in the proximal than the distal regions.

In mice, the Cyp3a family genes were primarily detected in liver and intestine. Liver expresses high levels of at least six out of eight Cyp3a isoforms Cyp3a11, 3a16, 3a25, 3a41a/b, 3a44 and 3a59 in mice. Whereas, Cyp3a11 is the most highly expressed Cyp3a in intestine compared to other Cyp3a's, expression of Cyp3a13 is higher in intestine than liver, and Cyp3a59 is higher in testis than liver. Similar to humans, mouse

hepatic Cyp3a exhibit sexual dimorphism with females expressing predominantly Cyp3a16, 41a/b, 3a44 and 3a59.(Renaud, Cui, Khan, & Klaassen, 2011).

Regulation of CYP3A

Nuclear hormone receptors (NR) such as the pregnane X receptor (PXR) (or NR subfamily 1, group 1, member 2, NR1I2), constitutive androstane receptor (CAR) (or NR subfamily 1, group 1, member 3, NR1I3) and VDR (or NR subfamily 1, group 1, member 1, NR1I1) are known to play a significant role in the regulation of CYP3A genes. PXR and CAR are regarded as xenosensors regulating detoxification and elimination of xenobiotics or toxic endogenous substances from the body (Mackowiak & Wang, 2016). Interestingly the other NR1 family member VDR also regulates numerous drug-metabolizing enzymes (Pavek & Dvorak, 2008). Unlike VDR, PXR is highly expressed in liver, but also in intestine and to a lesser extent in kidney (Pavek, 2016). Studies have reported that PXR is involved in the regulation of other CYPs such as CYP2B6, 2C9, 2C29, 3A5, 3A7 and transporters such as UGT1A1, P-glycoprotein (Pavek & Dvorak, 2008).

As suggested by recent studies PXR and CAR have the potential to cross-regulate CYP3A expression by different mechanisms either by sharing some ligands which could result in dual activation or binding of PXR and CAR to common target DNA response elements. But it has been reported that PXR is a dominant regulator of CYP3A expression compared to CAR (Goodwin, Hodgson, & Liddle, 1999).

Endogenous compounds and xenobiotics that are substrates to these NR's regulate CYP3A expression and contribute to huge inter individual variability in humans. Numerous other factors including genetic and environmental could contribute to the inter-individual variability of intestinal CYP3A4 but cannot explain it completely. Many studies reported clinically observed variability in intestinal CYP3A expression (Lown et al., 1994; Paine et al., 2005; Xu et al., 2006). Pharmacokinetics and pharmacodynamics of many drugs that are CYP3A4 substrates can be influenced by this variability (Gorski et al., 1998; Thummel et al., 1996) which can influence the extent of drug absorption, metabolism and elimination thereby altering the therapeutic efficacy or toxic response of the drug.

Substrates and Inducers of CYP3A

Substrates

The CYP3A family of enzymes are known to metabolize a variety of clinical drugs including macrolide antibiotics such as rifampin and naturally occurring and synthetic glucocorticoids such as dexamethasone (DEX). However, xenobiotic exposure can result in enhanced or repressed transcription of both intestinal and hepatic CYP3A.

Many of CYP3A substrates can also induce CYP3A expression. For example, DEX is both substrate and inducer of CYP3A4.

Inducers

Steroid hormones including glucocorticoid agonists and antagonists, macrolide antibiotics, imidazole antifungals, phenobarbital, polychlorinated biphenyls and organic pesticides are known to induce CYP3A transcription (Gonzalez, Liu, & Yano, 1993). Drugs such rifampin, phenobarbital, glucocorticoids can induce mouse Cyp3a11, Cyp3a13 and human CYP3A4 in vivo and in cultured hepatocytes (Schuetz, Wrighton, Barwick, & Guzelian, 1984; Watkins et al., 1985; Wrighton et al., 1985; Yanagimoto, Itoh, Sawada, & Kamataki, 1997). The CYP2B family genes are also normally induced by many of these same drugs (Honkakoski & Negishi, 1998; Strom et al., 1996). These interactions lead to clinically important drug-drug interactions when these inducer drugs are co-administered with CYP substrates (such as cyclosporin A, midazolam etc.).

Species Differences

Marked species differences exist regarding CYP3A induction. For example, in humans, rifampin causes a 10-fold induction of CYP3A (Strolin Benedetti & Dostert, 1994), whereas only a 2-fold induction in mice (Tredger, Smith, Powell-Jackson, Davis, & Williams, 1981). Pregnenolone-16 α -carbonitrile (PCN) is a strong inducer of rat CYP3A but not human.

Role of PXR, GR in CYP3A Induction

PXR is the main receptor involved in the steroid and drug induction of CYP3A (Kliwer et al., 1998). High concentrations of DEX and phenobarbital ligand activate both mouse and human PXR to induce CYP3A. There is no role for the glucocorticoid receptor (GR) in CYP3A induction. Glucocorticoids and anti-glucocorticoids follow the non-classic glucocorticoid induction pathway and induce CYP3A genes (Schuetz et al., 2000). Although glucocorticoids are both substrates and inducers of CYP3A enzymes, they have different requirements for the glucocorticoid receptor to induce CYP3A and CYP2B genes (Schuetz et al., 2000). In normal condition, they can bind to GR and induce genes regulated by GR. However, when glucocorticoid levels are extremely high (stressful conditions) or when high doses are given, they can produce maximum CYP3A induction through activation of PXR (Yusim, Franklin, Brooke, Ajilore, & Sapolsky, 2000). The dose required to activate PXR is usually at least 100-fold higher (Reid, 1997) than that required to activate GR. Physiologically, this feedback mechanism of PXR mediated CYP3A induction is to regulate the levels of corticosteroids and maintain steroid homeostasis to prevent any tissue damage during stressful conditions (H. Zhang et al., 1999) (Collier, Wu, & Pruett, 1998). The significant difference in the ligand binding domain (LBD) of PXR across species is responsible for the species differences in

induction of CYP3A – e.g., rifampin potently activates the LBD of human PXR, but is a weak activator of rat and mouse PXR, while PCN activates rodent PXR's LBD but not human PXR LBD.

(Schuetz et al., 1984) reported that GR might be involved in the synergistic induction of CYP3A by PCN and DEX. This synergistic induction could be a result of either protein-protein interaction between GR and PXR or inducers binding to glucocorticoid response elements (GRE) present upstream of PXR/RXR binding site on CYP3A gene. Moreover, recently it was reported that DEX treatment increased PXR and RXR mRNA accumulation in human hepatocytes likely through GR activation, which could also result in increased CYP3A expression (H. Zhang et al., 1999). However, induction of PXR and RXR mRNA requires their levels in hepatocytes to be rate limiting.

Dietary Interactions with CYP3A

Significant clinical interactions due to CYP3A induction or repression of activity by not only drugs but also dietary and natural compounds have been reported. For example, St. John's wort and grapefruit juice have been reported to alter CYP3A enzymes (Bailey, Malcolm, Arnold, & Spence, 1998; Bhardwaj et al., 2002; Johnson, Won, Kock, & Paine, 2017; Peltoniemi et al., 2012). Mechanistically, St. John's wort contains hyperforin one of the most potent ligand activators of PXR. Grapefruit juice contains 6,7-dihydrobergamottin that increases proteasome mediated degradation of intestinal CYP3A4 protein while having no effect on its mRNA. At least 20 drugs especially those with extremely low oral bioavailability due to pre-systemic metabolism were assessed to undergo interactions with grapefruit juice. Moreover, these interactions differ from patient to patient based on type and amount of grapefruit juice and individual susceptibility (Bailey et al., 1998). This indicates that dietary ingredients can also influence the level of CYP3A expression and therefore explain partly the huge interindividual variability in humans.

Similarly, vitamin D is also an important transcriptional regulator of CYP3A expression, and CYP3A is sensitive to changes in vitamin D levels. Consequently, vitamin D levels can also contribute to the CYP3A expression variability depending on the individual vitamin D status. These vitamin D-CYP3A interactions could be of significant importance specially when the co-administered drugs have a narrow therapeutic index or major toxicity.

Drug Transporters

The ATP-binding cassette (ABC) family of transporters are well known efflux transporters that transport wide range of drugs, exogenous chemicals and endogenous substances. P-glycoprotein (PGP) is a member of ABC subfamily B member 1 (ABCB1) which is present in multiple tissues but highly expressed in intestine (epithelial cells), liver (hepatocytes) brain, kidney (Allen, Brinkhuis, Wijnholds, & Schinkel, 1999). It

plays a significant role in protecting our body from toxins. Similar to CYP3A family, transporters also have a protective function by reducing the absorption of toxic chemicals, and hence there is a lot of substrate overlap between drug metabolizing enzymes and transporters (Borst & Schinkel, 2013), (Cascorbi, 2011). Dexamethasone, dasatinib, cyclosporin etc. are substrates of both CYP3A and PGP (Schinkel, Wagenaar, van Deemter, Mol, & Borst, 1995) (Schinkel et al., 1995).

Breast cancer resistant protein (BCRP) or ABC subfamily G2 member (ABCG2) is an efflux transporter mainly expressed in placenta, small intestine, liver, kidney, blood brain barrier and breast (Stacy, Jansson, & Richardson, 2013). BCRP transports many endogenous substances and a diverse groups of exogenous chemicals such as tyrosine kinase inhibitors, anthracene drugs, statins, and proton pump inhibitors, etc. (Mao & Unadkat, 2015; Meyer zu Schwabedissen & Kroemer, 2011).

Multidrug resistance associated protein (MRP4) or ABC subfamily C member 4 (ABCC4) is another well-known transporter that transports many endogenous and exogenous substances. It plays a significant role in renal excretion of its substrates (Russel, Koenderink, & Masereeuw, 2008).

Drug metabolizing enzymes and transporters are major routes of detoxification for the drugs in the body and also share substrates and inhibitors. Therefore, they contribute significantly to the drug-drug interactions and evaluating their expression in vitamin D deficiency condition could provide insights into mechanisms involved in vitamin drug interactions.

Vitamin D Deficiency

Vitamin D deficiency (VDD) is very common in a significant number of people worldwide and is considered endemic in many parts of the world with deficiency rates ranging from 42 to 61% in pediatric and adolescent population in the United States (J. Kumar, Muntner, Kaskel, Hailpern, & Melamed, 2009). Although lack of exposure to sunlight and low vitamin D intake are considered to cause vitamin D deficiency, clinically impaired intestinal vitamin D absorption or treatment with drugs enhancing vitamin D metabolism can also result in VDD (Glorieux & Pettifor, 2014; Prentice, 2013; Schafer et al., 2015). Vitamin D deficiency could lead to many disorders such as type II Rickets, increased bone fracture, etc.

Therefore, vitamin D supplementation is generally given to maintain vitamin D levels and to increase the bone mineral density in patients. Although many clinical trials investigated the effect of vitamin D supplementation with or without calcium to reduce fracture incidence, there is no consensus on the benefit of supplementing vitamin D in these patients (Bischoff-Ferrari et al., 2012; Lips, Gielen, & van Schoor, 2014). Furthermore, there is a concern that vitamin D supplementation could cause vitamin drug interactions with CYP3A4 substrates.

Vitamin D – CYP3A4 Clinical Interactions

Vitamin D (VD₃) deficiency leads to bone loss, decreased bone mineral density, rickets, osteoporosis, hyperparathyroidism and increased risk of fracture (Greer, 2009). Glucocorticoids such as DEX and prednisone are widely used clinically as part of leukemia chemotherapy. Long term exposure to glucocorticoids is known to produce adverse skeletal effects due to early rapid bone resorption and a late impaired bone formation (Mazziotti, Giustina, Canalis, & Bilezikian, 2007). This adverse effect is also attributed to increased VD₃ metabolism which can be detrimental to the VD₃ deficient leukemia patients.

Vitamin D – Intestinal CYP3A4

Since intestinal CYP3A4 mediated metabolism contributes to decreased oral bioavailability of its substrates, co-administration of VD₃ (a CYP3A4 inducer) with a CYP3A4 substrate might cause incomplete oral bioavailability of that drug substrate. On the other hand, vitamin D deficiency could increase the oral bioavailability of CYP3A4 substrates. A study by Schwartz et al., reported increased clearance of a CYP3A4 substrate, atorvastatin in vitamin D deficient humans supplemented with VD₃ (Schwartz, 2009). Another study reported seasonal variation in serum levels of CYP3A4 substrates, tacrolimus and sirolimus that mirrored the seasonal variation in serum levels of VD₃ (Lindh, 2011). Previously our lab has reported the seasonal variation of in vivo intestinal CYP3A4 expression, correlating with seasonal VD₃ levels (Thirumaran et al., 2012).

Hence, serum VD₃ levels, which is associated with intestinal CYP3A4 activity, has the potential to alter oral drug absorption and clearance. At St. Jude Children's Research Hospital, CYP3A4 substrates such as DEX and dasatinib are administered orally as part of chemotherapy to ALL patients (Christopher et al., 2008; Gentile, 1996).

Dexamethasone (DEX) is a synthetic analog of cortisol used during the induction, delayed intensification, and maintenance phases of ALL chemotherapy, most pediatric patients receive it for about half of their 2-3 years of treatment period (Margolin JF, 2006). Because DEX is both a substrate and inducer of CYP3A, understanding the effect of VD₃ deficiency on DEX systemic exposure and evaluating any vitamin drug interactions is necessary.

Dasatinib is a tyrosine kinase inhibitor, specifically administered to acute lymphoblastic leukemia patients with the BCR-ABL-translocation that generates the BCR-ABL+ oncogene that has elevated ABL kinase activity. Dasatinib can also inhibit SRC kinase which is involved in growth and progression of many cancers. Dasatinib is specifically approved to be used in imatinib resistant chronic myeloid leukemia and acute lymphoblastic leukemia. The oral bioavailability of dasatinib is very low ranging from 14% in mouse to 34% in dogs. Dasatinib undergoes high first-pass metabolism by CYP3A, both in the intestine and liver (Christopher et al., 2008). Any changes in

intestinal CYP3A due to vitamin D status might decrease or increase the pre-systemic metabolism of dasatinib in the gut, thereby influencing its pharmacokinetics.

Because both these drugs are administered orally, we evaluated whether changes in pharmacokinetics and clearance of dexamethasone and dasatinib occurred due to VD₃ deficiency.

In summary, small intestine is the first site of exposure to orally administered drugs where abundant drug metabolizing CYP3A enzymes and transporter are expressed. They are responsible for increasing or decreasing the oral bioavailability of many drugs. Because CYP3A4 has broad substrate specificity, many drugs have the potential to interact with CYP3A4 and cause drug-drug interactions. Most of these interactions have been studied and reported. In spite of many reports suggesting vitamin D deficiency in normal population as well as patients with various cancers, very little is known about vitamin D's interaction with CYP3A4 substrates. Because pediatric acute lymphoblastic patients are orally administered DEX and dasatinib, substrates of CYP3A4, it was important to determine whether supplementation of vitamin D to VD₃ insufficient/deficient patients might cause a drug interaction with either substrate or compromise anti-leukemic efficacy.

CHAPTER 2. VITAMIN D AND LEUKEMIA

Vitamin D in Health

Vitamin D regulates many different genes, most of which play an important role in physiological calcium homeostasis or bone formation. These genes include osteocalcin, osteopontin, parathyroid hormone (PTH) (Berridge, 2015). In intestine, $1,25(\text{OH})_2\text{VD}_3$ is known to regulate many processes such as intestinal calcium and phosphorus transport, nuclear events such as chromatin template activity, DNA-dependent RNA polymerase II activity and Ca^{+2} dependent ATP and alkaline phosphatase activities (Carlberg & Campbell, 2013). Although maintaining calcium and phosphate homeostasis is an important function of $1,25(\text{OH})_2\text{VD}_3$, many studies have demonstrated its role in other cellular processes such as proliferation, differentiation, immune modulation in both normal and malignant cells (Berridge, 2015; Bikle, 2012).

Vitamin D in Disease

Vitamin D promotes enterocyte differentiation and intestinal calcium and phosphorus absorption which helps in bone mineralization. Therefore, vitamin D deficiency can significantly affect the bone mineral density both by reduced absorption and enhanced resorption of calcium resulting in several disorders such as rickets and osteomalacia. CYP27B1 converts $25(\text{OH})\text{VD}_3$ to $1,25(\text{OH})\text{VD}_3$, and mutations in this gene resulting in inactive or deleted CYP27B1 cause vitamin D deficiency rickets type I (VDDR1) irrespective of normal vitamin D intake. Hereditary vitamin D resistant rickets (HVDDR or VDDR2) is another form of rickets where there is organ-resistance to vitamin D due to heterogenous loss of function mutations in VDR (Sahay & Sahay, 2012). Because calcium and bone homeostasis are highly interlinked, studies in both mice or humans lacking VDR or CYP27B1 has shown phenotype of rickets and osteomalacia. In spite of differences in role of vitamin D between human and mouse models, animal models could still help unravel complex mechanistic pathways involving similar genes that could affect humans (Christakos et al., 2016).

Epidemiological studies suggested a role of vitamin D not only in the skeletal system but also in cancer, cardiovascular disease, kidney disease, psoriasis etc. (Sahay & Sahay, 2012). Many studies have reported that vitamin D deficiency is associated with cardiovascular pathology. In humans, low vitamin D levels are associated with arterial hypertension, diabetes mellitus and dyslipidemia (Podzolkov, Pokrovskaya, & Panasenko, 2018). Because of vitamin D's role in various cellular processes such as proliferation, apoptosis, ageing of cells, it has been associated with various age-related diseases such as Alzheimer's, Parkinson's, multiple sclerosis diseases (Berridge, 2017). Although laboratory studies report extra-skeletal beneficial effects of supplementing vitamin D, it is important to obtain definite clinical data regarding the use of vitamin D and its analogues for treatment or prevention of disease processes.

Vitamin D in Cancer

Many cancer cells not only express VDR through which vitamin D exhibits its effects, but also express CYP27B1 or CYP24A1, the enzymes involved in vitamin D metabolism (Bikle, 2016). The expression of these enzymes can regulate the local production of active VD_3 , which can have significant implications in cancer progression (Hobaus, Thiem, Hummel, & Kallay, 2013; Swami et al., 2012). Several reports have suggested VDR expression in tumor cells to be a prerequisite for antineoplastic effects of $1,25-(OH)_2VD_3$ such as differentiation of HL60 leukemia cells into macrophages requires VDR expression (Abe et al., 1981).

Vitamin D Deficiency in Cancer

It has been reported that at least 72% of pediatric and young adults with cancer suffer vitamin D deficiency at diagnosis and this hypovitaminosis persists throughout the survivorship (Choudhary, Chou, Heller, & Sklar, 2013; Helou et al., 2014; Wallace et al., 2015). With significant proportions of cancer patients being vitamin D deficient, it became important to understand its association with the survival outcome. Several reports from pre-clinical as well as clinical studies show that vitamin D deficiency correlated with poor survival outcome. Vitamin D deficient mice inoculated with tumor cells such as colon or breast cancer had increase in tumor growth (Ooi et al., 2010; Ray et al., 2012; Tangpricha et al., 2005). On the other hand, repression of tumor formation was observed in rodents with sufficient vitamin D and calcium levels (Newmark et al., 2009).

In mouse xenograft models of prostate and breast cancer, it has been reported that vitamin D supplementation can inhibit tumor growth (Swami et al., 2012). In a pre-clinical study involving mice with pancreatitis, administration of calcipotriol, an analogue of vitamin D resulted in decreased fibrosis and inflammation. Induction of extensive stromal remodeling with reduced tumor-supportive signaling led to enhanced efficacy of co-administered chemotherapeutic agent and increased survival of the mice (N. Ding et al., 2013). In spite of vitamin D's (supplementation) ability to reduce tumor growth and metastasis, its adverse effects such as hypercalcemia hinder its clinical applicability.

Vitamin D Deficiency in Leukemia

One of the major factors leading to leukemia is perturbations in white blood cell (T or B cell) differentiation in the bone marrow (BM) due to a combination of genetic and environmental factors. Based on the lineage and persistence of the disease, leukemia is classified into four major types, acute lymphoblastic leukemia (ALL), chronic lymphoblastic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML). Considering the prevalence of VD_3 deficiency in leukemia patients, numerous researchers conducted studies to perceive the association of VD_3 and clinical outcome in different types of leukemia.

AML is characterized by immature hematopoietic myeloid precursors that have lost their ability to differentiate and proliferate normally (Tenen, 2003). Vitamin D deficiency is highly prevalent in AML patients with at least 80% of the patients at diagnosis have significantly low serum vitamin D levels (Seyedalipour et al., 2017) and has been associated with poor survival outcome whereas high vitamin D levels with better clinical outcome (Seyedalipour et al., 2017). AML cells, although expressing low constitutive levels of VDR, induced VDR expression at a post transcriptional level following treatment with 1,25-(OH)₂VD₃ (Gocek, Burska, Marchwicka, & Marcinkowska, 2012). In vitro studies using a variety of AML cell lines such as HL-60, AML-193, NB-4, U937 etc. indicated 1,25-(OH)₂VD₃ treatment induced differentiation of these cells into monocytes (Kim et al., 2012). *In vivo* studies using animal models with AML and treated with vitamin D analogues suggested significantly better survival of mice compared to the no treatment group (J. Y. Zhou et al., 1990). However, due to the limitations and inconsistencies between the studies, it is challenging to translate vitamin D or its analogues usage in the clinical setting (Cao et al., 2017).

Similar to AML, CLL patients also suffer with vitamin D insufficiency and that has been associated with poor overall survival outcome. Although higher 25-(OH)VD₃ levels show protective effect against development of CLL, no significant correlation between vitamin D levels and overall risk for lymphoid cancer development has been shown (Luczynska et al., 2013). In a study with 983 non-Hodgkin's lymphoma (NHL) patients comprised of subtypes including diffuse large B-cell lymphoma and T-cell lymphoma, 44% of patients were vitamin D deficient at the time of diagnosis (Drake et al., 2010). These insufficient serum vitamin D levels were associated with shorter event free and overall survival specifically in these subtypes. No correlations were observed in other NHL subtypes (Bittenbring et al., 2014).

These studies indicate the importance of measuring vitamin D levels at diagnosis, throughout the treatment and evaluating if vitamin D has any effect on the clinical outcome.

Vitamin D Deficiency in ALL

Approximately 60% of ALL patients are vitamin D deficient (Young et al., 2018) and are highly prone to the adverse bone effects associated with chemotherapy. Persistent decline in bone mineral density is observed from diagnosis until off-therapy for as long as 10-years following diagnosis in ALL patients (Demirsoy et al., 2017; Kuhl et al., 2012). Reasons include vitamin D deficiency at diagnosis, leukemic cell infiltration, irradiation, toxicities associated with chemotherapies such as corticosteroids and methotrexate which are known to intensify bone resorption and decrease osteoblast function. Supportive care measures such as antiepileptic agents also negatively impact vitamin D levels by interacting with CYP3A4, an enzyme involved in vitamin D metabolism/inactivation (Heaney, 2005; J. Y. Lee, So, & Thackray, 2013).

Unlike other leukemia malignancies, association of vitamin D levels and survival outcome has not been studied in ALL. One of the reasons for this could be that B-ALL cells do not express VDR, which is considered to be a prerequisite for vitamin D's anti-neoplastic activity. There are at least two clinical trials ((Dana Farber and Children's Hospital of LA (ClinicalTrials.gov Identifier number: NCT01574274 and NCT01317940, respectively) administering vitamin D to insufficient and deficient pediatric acute lymphoblastic leukemia patients to restore VD₃ sufficiency and determine if there is improved bone mineral density in the context of changes in body composition and body fat. In another study children and young adult ALL patients were supplied with vitamin D to maintain their bone mineral density and it was shown to be safe and effective in (Young et al., 2018). Nevertheless, long term health outcomes commonly observed in ALL patients such as osteonecrosis, fracture rate, and overall survival outcome have not been reported.

Since a significant proportion of ALL patients are VD₃ insufficient/deficient, it is critical to evaluate the role of vitamin D status in the progression of such disorders. Therefore, we aimed to investigate the effect of VD₃ on survival from BCR-ABL ALL in a mouse model. BCR-ABL is a fusion gene, also called Philadelphia chromosome (Ph⁺), that arises from the translocation between chromosomes 9 and 22 in at least 3-5% of pediatric ALL cases and 25% of adult ALL cases (Williams & Sherr, 2008). The resulting BCR-ABL oncogene has much higher ABL kinase activity.

Role of Vitamin D in the Bone Marrow Microenvironment

ALL is characterized by abnormal expansion of malignant lymphoblasts that replace hematopoietic stem cells (HSCs) from the bone marrow microenvironment (BMM) where they reside with a multitude of other cell types which help maintain homeostasis. HSCs tend to localize in two different niches in the bone marrow (BM), the perivascular niche and the endosteal niche (Nombela-Arrieta et al., 2013). Leukemia cells can migrate to the BMM similar to the HSCs (Peled et al., 1999), and can further disrupt the healthy microenvironment in the BM by creating a leukemia niche. This niche helps leukemia cells to proliferate better and hide from treatment and immune response (Fujisaki et al., 2011) (Nakasone et al., 2012).

The cells in the niche produce various cytokines and cell adhesion molecules that support lymphocyte proliferation and migration. Lymphocytes are attracted by a chemokine called stromal cell-derived factor 1 (SDF-1 or CXCL12) which is produced by various cells in the BMM (Peled et al., 1999; van den Berk et al., 2014). CXCL12 binds to CXCR4, a receptor expressed on the lymphocytes and attract them towards the BMM. This CXCR4-CXCL12 axis has been shown to be important in homing of ALL cells towards the BMM (Colmone et al., 2008; Juarez et al., 2007; Sipkins et al., 2005). Various cell types such as perivascular stromal cells, mesenchymal stem cells (MSCs), endothelial cells, osteoblasts, fibroblasts secrete CXCL12 (although to various degrees) to regulate HSC maintenance (L. Ding, Saunders, Enikolopov, & Morrison, 2012). Apart from these, CXCL12 abundant reticular cells (CARs) which can differentiate into

osteoblasts and adipocytes also produce a significant amount of CXCL12 to help maintain HSC self-renewal, proliferation, and migration (Omatsu et al., 2010).

Human marrow-derived stromal cells (hMSCs) or mesenchymal stem cells include progenitors of several lineages including osteoblasts, chondrocytes and adipocytes (Krebsbach, Kuznetsov, Bianco, & Robey, 1999; Prockop, 1997). Studies show that treatment of hMSCs with 1,25-(OH)₂VD₃ resulted in increased differentiation of hMSCs towards osteoblasts (P. Liu, Oyajobi, Russell, & Scutt, 1999). Therefore, we investigated if vitamin D status altered the expression of CXCL12, produced by these cells in the BMM which can play a huge role in proliferation and homing of the leukemia cells.

Vitamin D is well known to play an important role in bone homeostasis. In diseases such as pancreatitis and hepatic stellate cancer, vitamin D was shown to remodel the stromal cells with reduced tumor supportive signaling (N. Ding et al., 2013). Any changes in the bone marrow composition due to vitamin D deficiency can lead to bone marrow remodeling. Changes in expression of soluble factors such as chemokines, adhesion molecule in the bone could in turn alter the bone marrow microenvironment.

Considering VD₃ deficiency causes a significant bone loss and decreased bone mineral density, it becomes critical to ascertain if vitamin D status can remodel the BMM. Here, we aimed to evaluate the changes associated with vitamin D deficiency on the bone marrow microenvironment. We investigated the effect of VD₃ on both proportions of various BM cell types as well as expression of chemokines regulating tumor cell proliferation and homing to the BM that can ultimately impact the survival outcome.

Central Hypothesis

Vitamin D insufficiency or deficiency is common worldwide in the pediatric population, including those with leukemias. In addition, chemotherapies used to treat leukemia's such as glucocorticoids cause a further erosion of VD₃ through induction of CYP3A4, that metabolizes 1,25-(OH)₂VD₃ and 25-(OH) VD₃. Given the prevalence of VD₃ deficiency in the pediatric leukemia population, clinical trials are ongoing at multiple pediatric leukemia centers (Dana Farber Pediatric ALL, Children's Hospital LA) to determine whether VD₃ supplementation can restore VD₃ levels and help improve bone mineral density. However, there is a concern that VD₃ treatment might cause a vitamin-drug interaction with CYP3A4 substrates because

- VD₃ is known to transcriptionally induce intestinal CYP3A4 (Thummel et al., 2001)
- Intestinal CYP3A4 shows seasonal variation (Thirumaran et al., 2012) and

- Schwartz JB et al., reported that VD₃ supplementation increased atorvastatin (CYP3A4 substrate) clearance in VD₃ deficient patients (Schwartz, 2009).

Although many reports suggest an association between VD₃ deficiency and poor survival outcome in various leukemia subtypes, there is no data on the potential effect of VD₃ on survival of mice from pre-B cell leukemia.

Therefore, it is imperative to determine both the effect of VD₃ on intestinal CYP3A expression and the pharmacokinetics of orally administered CYP3A4 metabolized leukemia therapies and its effect on the efficacy of those therapies on survival from acute lymphoblastic leukemia (ALL). Upon conclusion, we will understand the consequences of VD₃ deficiency on survival outcome in a pre-B cell leukemia and interactions of VD₃ with chemotherapeutics that are CYP3A4 substrates.

The central hypothesis of this proposal is “VD₃ deficiency causes vitamin-drug interactions with antileukemic - CYP3A4 substrates, and also affects leukemia cell proliferation and migration leading to differential homing pattern causing an impact on survival outcome in BCR-ABL+ Arf^{-/-} acute lymphoblastic leukemia”. Below specific aims (**Figure 2-1**) were designed to evaluate this hypothesis.

Specific Aim 1 (Chapter 3)

- a. Investigate the effect of VD₃ status on expression of intestinal and hepatic mouse Cyp3a's, human CYP3A4 and other ADME genes in intestine, liver and kidney.
- b. Determine if VD₃ status alters the pharmacokinetics of orally administered anti-leukemic-CYP3A substrates, dexamethasone and dasatinib.

Specific Aim 2 (Chapters 3 and 4)

To evaluate the effect of VD₃, alone or in combination with dexamethasone on survival of BCR-ABL+ Arf^{-/-} ALL mouse model.

Specific Aim 3 (Chapter 4)

To determine the effect of VD₃ on the bone marrow microenvironment remodeling, and BCR-ABL Arf^{-/-} leukemic blast migration, homing and proliferation using *in vivo* and *in vitro* models.

Figure 2-1. Schematic for specific aims.

CHAPTER 3. VITAMIN D STATUS ALTERS DRUG DETOXIFICATION GENE EXPRESSION AND DEXAMETHASONE INDUCTION OF CYTOCHROME P450S IN MICE

Introduction

The Cyp3a subfamily of enzymes are responsible for the metabolic transformation of many drugs and some endogenous molecules. CYP3A4, an important member of Cyp3a family in humans can metabolize at least 50% of the prescription drugs and is found to be highly expressed in drug detoxification organs such as liver and small intestine (Kolars et al., 1994). Intestinal CYP3A-mediated first-pass metabolism of orally administered substrates such as cyclosporine, midazolam, lovastatin, felodipine, saquinavir, and buspirone is a major basis for their low oral bioavailability (Kolars, Awni, Merion, & Watkins, 1991) (Paine et al., 1997).

Induction of hepatic and intestinal CYP3A4 is the basis for many drug-drug interactions. CYP3A4 is induced by nuclear hormone receptors in the NR1I family that are each activated by specific ligands: NR1I2/Pregnane X receptor (PXR) (e.g., glucocorticoids, rifampicin) and NR1I3/constitutive activated receptor (CAR) (e.g., phenobarbital) (Chai, Cherian, Wang, & Chen, 2016) (Matsunaga et al., 2012) (Lin, 2006). We previously determined that CYP3A4 is also induced by NR1I1/Vitamin D receptor (VDR) activated by 1,25 (OH)₂VD₃ (Thummel et al., 2001), although this induction occurs primarily in intestinal enterocytes where VDR is highly expressed versus hepatocytes where it is lowly expressed (Matsubara et al., 2008).

Several lines of evidence suggest vitamin D could cause a vitamin-drug interaction (VDI) with CYP3A4. (Lindh, 2011) showed in humans that systemic exposure of the immunosuppressant CYP3A4 substrates sirolimus and tacrolimus varied seasonally (higher in winter than summer), correlating with sunlight exposure and vitamin D levels. We showed that human intestinal biopsy CYP3A4 expression varied seasonally with sunlight and vitamin D levels (higher in Spring/Summer and lower in Fall/Winter) and corresponded with a higher systemic midazolam level in patients dosed in winter versus summer (Thirumaran et al., 2012). (Schwartz, 2009) showed that vitamin D deficient patients given vitamin D supplementation had increased clearance of the CYP3A4 substrate atorvastatin.

Vitamin D deficiency or insufficiency is common in patients with acute leukemias (Naz, R, T, & Mahboob, 2013). Approximately 60% of acute lymphoblastic leukemia (ALL) patients at diagnosis are vitamin D insufficient or deficient, with deficiency and insufficiency more common in winter than summer (Young et al., 2018). Vitamin D deficiency in leukemia patients in combination with long term glucocorticoid therapy leads to reduction in bone mineral density (BMD) (Demirsoy et al., 2017). Some of these patients are prescribed VD₃ supplementation to maintain VD₃ levels and restore BMD (Orgel et al., 2017). Although intestinal Cyp3a expression can be regulated by VD₃ and many chemotherapeutic agents are Cyp3a substrates, it has not yet been determined

whether vitamin D sufficiency versus deficiency would affect the systemic concentrations of these chemotherapies. In this study we determined whether vitamin D sufficient versus deficient mice (a) differ in intestinal expression of VDR target genes, such as Cyp3a, or (b) differ in systemic exposure to the orally administered Cyp3a metabolized leukemia therapies DEX and dasatinib, or (c) differ in DEX's anti-leukemic efficacy.

Methods

Animals

Association for Assessment and Accreditation for Laboratory Animal Care (AALAC) guidelines were followed for all experimental animal procedures and housing, all protocols were approved by Institutional Animal Care and Use Committee (IACUC). E5-E6 pregnant C57BL/6J female mice were kept on the VD₃ deficient (Harlan catalog no. 5A69, 0 IU of vitamin D) and VD₃ sufficient diets, (catalog. No. 5BV8, 3.3 IU of vitamin D) to develop vitamin D deficient and vitamin D sufficient mice (**Figure 3-1**), respectively. Pregnant dams were maintained on these diets throughout pregnancy, and after parturition, and pups were maintained on the corresponding diets after weaning as described in (Annu et al., 2020). The pups were age matched and both male and female, VD₃ deficient and VD₃ sufficient mice were used for Cyp3a expression, pharmacokinetic and efficacy studies. For BCR-ABL survival studies with DEX treatment, C57BL/6J sufficient mice were placed on VD₃ deficient and VD₃ sufficient diets beginning at weaning.

To observe the effect of vitamin D status on hCYP3A4 expression, cryopreserved hCYP3A4 transgenic (tg) mouse sperm was used to re-derive the humanized CYP3A4 transgenic mouse model (Granvil et al., 2003). E5-E6 pregnant hCYP3A4-tg female mice were kept on VD₃ deficient and sufficient diets throughout pregnancy and after parturition as described for C57BL/6J mice. Pups were maintained on the corresponding diets after weaning and were euthanized at 8 and 12 weeks of age for tissues, blood was collected by cardiac puncture and serum isolated and stored at -80°C for 25-OH-VD₃ analysis. Vitamin D deficiency in the mice was confirmed by analyzing serum 25(OH)VD₃ levels in randomly selected mice in all studies (Table 1) and sent to the Veterinary Diagnostic Laboratory at Michigan State University (Lansing, MI) for 25-(OH) VD₃ analysis. Intestine (duodenum), liver and kidney were harvested and stored in RNA later at -20°C.

mRNA Quantification by Real-time PCR

Mouse intestine (duodenum), liver and kidney tissues stored in RNA later at -20°C were used for RNA isolation. Trizol (Ambion Life technologies, CA, USA or Thermo Fisher scientific, CA, USA) was used to extract RNA according to the

Figure 3-1. Design to develop age-controlled vitamin D-sufficient and -deficient mice.

manufacturers protocol. Sterile Omni tips (OMNI International, GA, USA) were used to homogenize the tissue using Omni tissue homogenizer. 500 ng of RNA was used to generate cDNA using the ThermoscriptTM RT-PCR system (Invitrogen ThermoFisher, CA, USA). The cDNA was diluted to a total volume of 70 µL. Primers used for the amplification:

mCyp3a11 (F) CTGGGCCCCAAACCTCTGCCA
mCyp3a11 (R) TGTGACAGCAAGGAGAGGCGT

mCyp3a13 (F) TACCCCAGTATTTGATGCAC
mCyp3a13 (R) AGATAACTGACTGAGCCACA

mMdr1a (F) TGCCACCACGATCGCCGAGA
mMdr1a (R) TCAGCTGCGCCCCTCTCTCA

mBcrp (F) CCATAGCCACAGGCCAAGT
mBcrp (R) GGGCCACATGATTCTTCCAC

hCYP3A4 (F) CACAGATCCCCCTGAAATTAAGCTTA
hCYP3A4 (R) AAAATTTCAGGCTCCACTTACGGTG

mCyp2b10 (F) CTTGCGCCTGCTGGAGCTGT
mCyp2b10 (R) GGTCCAAGGTGGCCCTGTGC

Gapdh (F) ACCACAGTCCATGCCATCAC
Gapdh (R) TCCACCACCCTGTTGCTGTA

Villin (F) AGGAAGGAGGAGCACCTGTC
Villin (R) GGACAGCCAGAGAGCTTCAA

Real-time PCR quantification was performed using SYBR GreenERTM qPCR supermix for ABI PRISM Instrument (Life Technologies, Grand Island, NY) according to manufacturer's instructions. Samples were run at the following conditions: 95°C for 15 min, 40 cycles of 92°C for 30 sec, 60°C for 30 sec, 68°C for 1 min followed by a dissociation step. The relative amounts of mRNAs in each sample were normalized to Villin or Gapdh for intestine samples or Gapdh values for liver, kidney samples to control for quality of the mRNA. Comparative CT (ddCt) method was used to determine quantitative PCR values.

Dexamethasone Pharmacokinetics

Dexamethasone administered in drinking water

8-10-week-old C57BL/6 VD₃ Sufficient and VD₃ deficient, male (n=8-13) and female (n=5) mice were treated with the discontinuous DEX regimen as described in

(Ramsey et al., 2015). 8 mg/L of dexamethasone sodium phosphate (Fresenius kabi LLC, Lake Zurich, IL, USA) is given in drinking water for 3.5 days followed by OFF dexamethasone but 600 mg/L of Sulfamethoxazole (Aurobindo Pharma Inc, Dayton, NJ, USA) and 120 mg/L of trimethoprim (Aurobindo Pharma Inc, Dayton, NJ, USA) for the next 3.5 days. 1 g/L of tetracycline (Sigma-Aldrich Inc, St. Louis, MO, USA) was added during both phases. Assuming that mice drink 5 mL of water per day, the dose they receive would be 1.6-2 mg/kg/day (Ramsey et al., 2015). This discontinuous DEX ON/OFF schedule is then repeated with blood samples that were collected on day 0 (basal), and after each pulse of DEX i.e., day 3.5, 10.5 and 17.5 during the discontinuous DEX treatment (**Figure 3-2**). Blood was centrifuged at 10000 rpm for 2 minutes and plasma was stored on dry ice. At the end of *in vivo* procedures, all plasma samples were transferred from dry ice and stored at -80°C until analysis. DEX plasma concentrations were measured using liquid chromatography with tandem mass spectrometric detection (Ramsey et al., 2015).

Single oral dexamethasone gavage

The dosing and sampling scheme to assess DEX PK in mice after a single oral dose were performed as previously described (J. Li et al., 2018). Briefly, 14-week-old C57BL/6 VD_3 sufficient and deficient male ($n=5$) and female ($n=5$) mice were given 2 mg/kg dexamethasone sodium phosphate dissolved in 0.5% hydroxypropyl-methylcellulose (HPMC) and 1% Tween 80 by oral gavage. Blood was collected via saphenous/retro-orbital vein at time points: 0.25, 0.75, 0.5, 1, 1.5, 2, 4, 8, 16 hours after treatment (**Figure 3-3**). Samples were spun at 10000 rpm for 2 minutes for plasma separation and stored at -80°C until further analysis. DEX plasma concentrations were measured using liquid chromatography with tandem mass spectrometric detection (Ramsey et al., 2015).

Dasatinib Pharmacokinetics

The total plasma pharmacokinetics of dasatinib in C57BL/6 VD_3 sufficient and deficient mice were assessed after single oral gavage of 10 mg/kg of dasatinib free base equivalents (St. Jude Compound Management, SJ000518976-9, LC Labs, D-3307, Lot BDS-106, purity > 99%) was dissolved in 80mMol/L sodium citrate buffer pH 3.1 for a total Dasatinib concentration of 1 mg/mL for a 10 mL/kg gavage volume. Mice were grouped by vitamin D status and sex and studied at 8 and 10 weeks of age. Survival saphenous and facial bleeding of mice was conducted using IACUC-approved methods at 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 and 24 hr. post dose, with 3 mice per time point (**Figure 3-4**). Each mouse was sampled 3 times on 2 separate occasions of dasatinib dosing. Blood was collected into a Sarstedt Minivette POCT 50 μL K3 EDTA capillary device, dispensed into a microtube and vortexed to mix the anticoagulant. The tubes were then immediately centrifuged to plasma and stored on dry ice for the remainder of the study. At the end of the *in vivo* procedures, plasma samples were transferred from dry ice and

Figure 3-2. Experimental design for pharmacokinetics of DEX using discontinuous DEX regimen in drinking water.

Data source: Ramsey, L. B., Janke, L. J., Payton, M. A., Cai, X., Paugh, S. W., Karol, S. E., . . . Relling, M. V. (2015). Antileukemic Efficacy of Continuous vs Discontinuous Dexamethasone in Murine Models of Acute Lymphoblastic Leukemia. PLoS One, 10(8), e0135134. [doi:10.1371/journal.pone.0135134](https://doi.org/10.1371/journal.pone.0135134)

Figure 3-3. Experimental design for pharmacokinetics of DEX post oral DEX gavage (2 mg/kg).

Data source: Li, J., Chen, R., Yao, Q. Y., Liu, S. J., Tian, X. Y., Hao, C. Y., . . . Zhou, T. Y. (2018). Time-dependent pharmacokinetics of dexamethasone and its efficacy in human breast cancer xenograft mice: a semi-mechanism-based pharmacokinetic/pharmacodynamic model. Acta Pharmacol Sin, 39(3), 472-481. [doi:10.1038/aps.2017.153](https://doi.org/10.1038/aps.2017.153)

Figure 3-4. Experimental design for pharmacokinetics of dasatinib post oral dasatinib gavage (10 mg/kg).

Data source: Furmanski, B. D., Hu, S., Fujita, K. I., Li, L., Gibson, A. A., Janke, L. J., . . . Baker, S. D. (2013). Contribution of ABCC4-mediated gastric transport to the absorption and efficacy of dasatinib. *Clin Cancer Res*, 19(16), 4359-4370.
[doi:10.1158/1078-0432.CCR-13-0980](https://doi.org/10.1158/1078-0432.CCR-13-0980)

placed at -80°C until analysis. Dasatinib bioanalysis and pharmacokinetic analysis (see below) were performed by Preclinical Pharmacokinetic Shared Resource at St. Jude Children's Hospital and described in supplementary methods.

Dasatinib bioanalysis

Total plasma Dasatinib concentrations were assessed using a sensitive and specific liquid chromatography, tandem mass spectrometry assay. Dasatinib stock solutions were prepared in acetonitrile and used to spike matrix calibrators and quality controls. Protein precipitation was performed using a 1:4 ratio of plasma to 15 ng/mL erlotinib HCl (St. Jude Compound Management, S00004053, Inventory ID: SJCH0042996, Purity > 95%) in methanol as an internal standard. A 3µL aliquot of the extracted supernatant was injected onto a Shimadzu LC-20ADXR high performance liquid chromatography system via a LEAP CTC PAL autosampler. The LC separation was performed using a Phenomenex Kinetex 2.6 µm EVO C18 (100A, 50 x 2.1 mm) maintained at 50 C with gradient elution at a flow rate of 0.5 mL/min. The binary mobile phase gradient began with a linear increase to 100% B in 4.0 minutes. The column was then rinsed for 2.0 minutes at 100% B and then equilibrated at the initial conditions for two minutes for a total run time of eight minutes. Under these conditions, the analyte and IS eluted at 1.81 and 2.21 minutes, respectively. Analyte and IS were detected with tandem mass spectrometry using a SCIEX API 5500 Q-TRAP in the positive ESI mode with monitoring of the following mass transitions: Dasatinib 488.20 → 401.00, erlotinib HCl 394.20 → 250.10.

The experimental bioanalytical runs were all found to be acceptable for the purpose of a singlicate non-GLP, preclinical PK assessment. A linear model (1/X² weighting) fit the calibrators across the 1.00 to 500 ng/mL range, with a correlation coefficient r^2 of ≥ 0.9058 . The lower limit of quantification (LLOQ), defined as a peak area signal-to-noise ratio of 5 or greater versus a matrix blank with IS, was 1.00 ng/mL. The intra-run precision and accuracy was < 11.6% CV and 90.6% to 107% respectively.

Pharmacokinetic analysis

Plasma concentration-time (Ct) data in ng/mL for dasatinib were grouped by individual mouse, Vitamin D status, sex, and age, and were analyzed using nonlinear mixed effect (NLME) modeling as implemented in Monolix version 2018R1 (Lixoft SAS, Antony, France). Briefly, parameters and the Fisher Information Matrix (FIM) were estimated using the stochastic approximation expectation maximization (SAEM) algorithm, and the final log-likelihood estimated with importance sampling, all using the default Monolix initial settings, except that 1000 iterations were permitted for estimation of FIM using stochastic approximation. A variety of models were fit to the dasatinib Ct data, parameterized using apparent clearances, volumes of distribution, and absorption rate constant as needed. These models were assessed for goodness of fit using the -2-log likelihood (-2LL) value, Akaike and Bayesian Information Criterion (AIC, BIC), visual predictive checks, plots of model individual and population predicted versus observed

data, residual plots, and the standard error of parameter estimates. A log-normal inter-individual and inter-occasion parameter distribution was assumed on selected supported parameters, with both on- and off-diagonal elements of parameter covariance matrices tested. Additive and/or proportional error models were tested and implemented as supported. Beal's M3 method was used to handle any data that were below the LLOQ or above the upper limit of the assay range. The grouping levels were tested as categorical covariates on supported PK parameters, primarily the apparent oral clearance (Cl). A covariate effect was considered significant if its addition reduced the -2LL by at least 3.84 units ($P < 0.05$, based on the χ^2 test for the difference in the -2LL between two hierarchical models that differ by 1 degree of freedom). Additionally, Wald test P values were outputted for each covariate effect by the Monolix software.

Survival Study

Murine BCR-ABL Arf^{-/-} Luc⁺ ALL cells were created by Dr. Charles Sherr (St. Jude Children's Research Hospital) and provided by Dr. Mary Relling (St. Jude Children's Research Hospital). Cells were cultured previously as described (Ramsey et al., 2014). C57BL/6 VD₃ sufficient (n=24) and deficient (n=22) male mice (8-18 wk of age) were injected with 2,000 BCR-ABL Arf^{-/-} Luc⁺ ALL cells retro-orbitally on day zero. Body weights were not significantly different at the start of the study (day 0). Starting on day 4, mice were kept ON the discontinuous DEX dosing regimen (described above) where DEX was supplied in drinking water for 3.5 days, and then removed for the next 3.5 days, and this discontinuous DEX ON/OFF schedule was repeated until the animals died from leukemia (**Figure 3-5**). Xenogen IVIS-200 (Caliper Life Sciences, Hopkinton, MA) was used to acquire bioluminescent images 5 minutes post-intra peritoneal injection of 200 μ L of 100 mg/kg D-Luciferin (Caliper Life Sciences, Hopkinton, MA). Mice were anaesthetized with isoflurane during this procedure. The bioluminescent signal (photons/s) from a fixed region of interest (ROI) is used to monitor the disease progression twice every week. Images acquired here were analyzed using Living Image 3.1 software (Caliper Life Sciences), where whole body luciferase flux measurements (photons/s) was quantified as disease burden. Euthanasia was performed when the animal reached moribund stage which was determined based on hind limb paralysis, scruffy coat, lethargy or an inability to obtain food or water. We repeated the survival study with DEX treatment by employing pups from pregnant females placed on VD₃ sufficient (n=13) and deficient (n=10) diets and observed similar effect that VD₃ deficient mice with DEX treatment survived better than VD₃ sufficient mice (data not included).

Data Analysis

Unpaired t-test or Mann-Whitney non-parametric tests were used to determine the significance in gene expression assays. Survival data was analyzed using Kaplan Meier

Figure 3-5. Experimental design for survival of mice from BCR-ABL ALL placed on discontinuous DEX regimen in drinking water.

Data source: Ramsey, L. B., Janke, L. J., Payton, M. A., Cai, X., Paugh, S. W., Karol, S. E., . . . Relling, M. V. (2015). Antileukemic Efficacy of Continuous vs Discontinuous Dexamethasone in Murine Models of Acute Lymphoblastic Leukemia. PLoS One, 10(8), e0135134. [doi:10.1371/journal.pone.0135134](https://doi.org/10.1371/journal.pone.0135134)

survival analysis and Gehan-Breslow-Wilcoxin test was used to determine significance. Significance is calculated at $p < 0.05$ and the Grubb's test on GraphPad QuickCals was used to remove any outliers.

Results

Impact of Vitamin D Deficiency on Murine Duodenum Cyp3a and Transporter mRNA Expression

Female C57BL/6 VD₃ deficient mice had lower Cyp3a11 expression than VD₃ sufficient mice, but the difference did not reach statistical significance (**Figure 3-6B**), while no difference in Cyp3a11 mRNA expression was observed between male VD₃ sufficient versus deficient mice (**Figure 3-6A**). To better predict the effect of VD₃ levels on human intestinal CYP3A4, we used a humanized transgenic mouse containing a bacterial artificial chromosome with the complete human CYP3A4 gene including all of its regulatory sequences (Cheung et al., 2006). Both mCyp3a11 and hCYP3A4 mRNA expression trended lower in male 8-week old (but not 12 week) VD₃ deficient versus sufficient hCYP3A4-tg mice (**Figure 3-6C**). However, Cyp3a11 and hCYP3A4 expression were significantly lower in VD₃ deficient female 12-week-old (but not 8 week) hCYP3A4-tg mice (**Figure 3-6E**, $*p < 0.05$). Because ABC efflux transporters play a major role in decreasing the oral bioavailability of DEX (Abcb1a/Mdr1a/P-glycoprotein) (Schinkel et al., 1995) and dasatinib (Mdr1a, Abcg2/Bcrp) (Hiwase et al., 2008) we evaluated their expression and found their mRNAs were significantly higher in the duodenums of VD₃ deficient mice compared to sufficient mice (**Figure 3-6C, F**, $*p < 0.05$, $**p < 0.01$). Vitamin D deficiency was confirmed in serum obtained from terminal blood samples by analysis of 25-(OH) VD₃ in the control, vitamin D deficient mice (**Table 3-1**).

Cyp and Transporter Expression in Liver and Kidney of VD₃-Sufficient and -Deficient hCYP3A4-tg Mice

Because VDR can regulate expression of Cyp and transporters in other tissues important for drug disposition, we examined expression of these detoxification genes in kidneys and liver of mice with different vitamin D levels. In male mice, VD₃ status had no significant effect on hepatic Cyp3a, while in females hCYP3A4 expression trended lower in VD₃ deficient mice (**Figure 3-7A, B**). Renal expression of hCYP3A4 was unchanged while Cyp24a1, a gene highly regulated by VDR (Kagi et al., 2018), was lower in both male and female VD₃ deficient mouse kidney (**Figure 3-7A**, $*p < 0.05$). In liver hepatic Mdr1a and Mrp4 were higher in male VD₃ deficient mice (**Figure 3-7C**), but significantly lower in female VD₃ deficient mice (**Figure 3-7D**, $*p < 0.05$, $**p < 0.01$). Renal Mdr1a and Mrp4 were lower in VD₃ deficient mice, while in female mice VD₃ levels had no significant effect on expression of renal efflux transporters (**Figure 3-7D**).

Figure 3-6. Cyp and transporter gene expression in duodenum of VD₃ sufficient and deficient mice.

Notes: Influence of VD₃ deficiency on intestinal Cyp3a11 expression in C57BL/6 (A) male, n=3 and (B) female, n=3 mice. Comparative expression of intestinal Cyp3a11, hCYP3A4, and drug efflux transporters in 8 and 12-week old hCYP3A4-tg (C, E) male and (D, F) female mice. Data represents the results from 4-6 independent mice per group in hCYP3A4-tg experiments and are expressed as mean (bars) + SE (error bars) fold change in mRNA expression in deficient mice (green bar) relative to each mRNAs expression in sufficient mice set as one (red bar). Villin was used as an internal control to normalize the target gene expression data. Mann-Whitney nonparametric test on GraphPad was used to determine significance between the groups (**p<0.01, *p<0.05).

Table 3-1. Serum 25(OH)VD₃ levels in VD₃-sufficient and -deficient mice (Representative date).

Sample name	Gender	25-(OH) VD ₃ (nmol/L)	Diet
C57BL/6	M	246	VD ₃ sufficient
C57BL/6	M	403	VD ₃ sufficient
C57BL/6	F	454	VD ₃ sufficient
hCYP3A4-tg	M	297	VD ₃ sufficient
hCYP3A4-tg	M	171	VD ₃ sufficient
hCYP3A4-tg	F	221	VD ₃ sufficient
hCYP3A4-tg	F	187	VD ₃ sufficient
C57BL/6	M	6	VD ₃ deficient
C57BL/6	M	17	VD ₃ deficient
C57BL/6	M	18	VD ₃ deficient
C57BL/6	F	7	VD ₃ deficient
C57BL/6	F	12	VD ₃ deficient
hCYP3A4-tg	M	15	VD ₃ deficient
hCYP3A4-tg	M	15	VD ₃ deficient
hCYP3A4-tg	F	17	VD ₃ deficient
hCYP3A4-tg	F	12	VD ₃ deficient

Notes: M = Male, F= Female.

Figure 3-7. Cyp and transporter gene expression in liver and kidney of 12-week old hCYP3A4 mice.

Notes: Comparative expression of hepatic and renal Cyps and efflux transporters in 12-week old hCYP3A4-tg (A, C) male and (B, D) female mice. Data was analyzed and graphed as described in **Figure 3-6** legend.

Plasma Dexamethasone Concentrations in VD₃ Deficient versus Sufficient C57BL/6 Mice

Next, the effect of VD₃ status on DEX systemic exposure was compared following a discontinuous oral DEX regimen, similar to that used to treat pediatric ALL patients, and that achieves plasma DEX concentrations (2.6 – 201.5 nM) in mice similar to those achieved in pediatric patients on a discontinuous DEX regimen (Ramsey et al., 2015). Male VD₃ deficient mice had significantly (**Figure 3-8A**, * $p < 0.05$) higher plasma DEX levels compared to sufficient mice with a mean of 31.7 nM (n=8) and 12.43 nM (n=11), respectively, on day 3.5, but no difference at later time points. A similar trend was observed in females (**Figure 3-8B**) with the mean plasma DEX concentration only different (63 nM in deficient versus 52 nM in sufficient mice) at an early time-point.

When we repeated the oral discontinuous DEX PK study in another 60 mice (15 per group) (**Table 3-2**) the DEX plasma concentrations were below the lower limit of assay detection in a majority of both male (80%) and female (68%) VD₃ sufficient mice from Days 3.5 + 10.5, but was measurable in all female VD₃ deficient mice and only below the limit of quantification in 8% of VD₃ deficient female mice. This suggested that the higher intestinal Cyp3a in VD₃ sufficient versus deficient mice was impacting DEX systemic exposure, but only at the earliest exposure time points.

Because some of the variability in DEX concentrations between mice in all groups could have resulted from the mice drinking variable amounts of water, DEX pharmacokinetics was investigated following a single oral gavage of DEX (2 mg/kg) in both male and female VD₃ sufficient and VD₃ deficient mice. There was no significant difference between male or female VD₃ sufficient versus deficient mice in their DEX systemic concentration at any single time point or in DEX area under the concentration curve (AUC) (**Figure 3-9**).

Tissue-specific Impact of Dexamethasone on Intestinal and Hepatic CYP Expression in C57BL/6 VD₃ Sufficient versus VD₃ Deficient Mice

Why was there an apparent time-dependent effect of VD₃ status on DEX PK in mice (difference between groups seen only at early time points) on the discontinuous DEX regimen? We hypothesized that VD₃ regulated changes in intestinal Cyp3a expression might be negated by prolonged treatment with DEX, that is also an inducer of Cyp3a. Therefore, we determined whether there was any difference in DEX induction of Cyp's in intestine and liver of VD₃ sufficient versus VD₃ deficient mice. Cyp expression was analyzed in intestine and liver tissue from mice at day zero (no DEX) and on day 3.5 after a pulse of DEX using the oral discontinuous regimen. Duodenal Cyp3a11, Cyp3a13 and Cyp3a44 mRNAs were significantly induced (4-6-fold) (**Figure 3-10A-C**, * $p < 0.05$) by DEX only in the VD₃ deficient mice. In the liver Cyp2b10 was induced at least 50-fold in both VD₃ sufficient and VD₃ deficient male and female mice, with no significant induction of hepatic Cyp3a's in any mice (**Figure 3-10D-G**, * $p < 0.05$). Thus, there were divergent

Figure 3-8. Plasma dexamethasone concentrations in C57BL/6 VD₃ sufficient and deficient mice on a discontinuous dexamethasone regimen.

Notes: Plasma DEX concentrations on days 0 (basal), and at the end of each pulse of DEX i.e., days 3.5, 10.5 and 17.5 in (A) male (n=8-13/group), and (B) female (n=5) VD₃ sufficient and VD₃ deficient mice. The t-test on GraphPad was used to determine significance between the groups (*p<0.05).

Table 3-2. Repeat dexamethasone PK in drinking water.

Group	Sex	Day 3.5	Day 10.5	Day 17.5
VD ₃ sufficient	M	5 (10/15 BLQ)	0 (10/10 BLQ)	5
VD ₃ deficient	M	13 (2/15 BLQ)	10	5
VD ₃ sufficient	F	5 (10/15 BLQ)	3 (7/15 BLQ)	5
VD ₃ deficient	F	15	10	5

Notes: Number of samples in each group with dexamethasone plasma levels that were measurable or below the lower limit of quantification (BLQ). M = Male, F= Female.

Figure 3-9. Plasma DEX pharmacokinetics in VD₃-sufficient and -deficient mice after a single oral gavage of 2 mg/kg dexamethasone.

Notes: Plasma DEX concentrations in VD₃-sufficient and -deficient 14-week (A) male and (B) female mice. A t-test was used to determine the significance of differences between VD₃-sufficient and -deficient mice.

Figure 3-10. Induction of duodenal and hepatic Cyps in VD₃-deficient mice after dexamethasone treatment.

Notes: Cyp3a mRNA expression was analyzed in VD₃-sufficient and -deficient C57BL/6 male mice at time zero and following Dex in drinking water for 3.5 days, n=3-5 per group: Intestinal (A) Cyp3a11, (B) Cyp3a13, (C) Cyp3a44, and hepatic Cyps in VD₃-sufficient and -deficient (D, F) male and (E, G) female mice (n=3-5 per group). Gapdh was used as an internal control to normalize the target gene expression data. Mann-Whitney nonparametric test on GraphPad was used to determine significance between the groups (**p<0.01, *p<0.05).

effects of DEX on induction of Cyp3a's and Cyp2b that was both tissue-specific: DEX induction of Cyp3a was limited to VD₃ deficient mice in intestine, and DEX induction of Cyp2b was observed in liver. A model is proposed in the discussion to account for these differential responses.

Dasatinib Pharmacokinetics (Oral Gavage) in C57BL/6 Mice

The plasma PK of dasatinib (a drug given throughout therapy to treat BCR-ABL+ acute lymphoblastic leukemias) was evaluated after two separate single oral gavage doses of 10 mg/kg to determine the effects of VD₃ status, sex and age (8 wk versus 10 wk) on plasma PK using a nonlinear mixed effects PK modeling approach. Blood samples were collected at 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 24 hr. post-dose with N=3 mice/group/time point and assayed using LC-MS. There was no influence of VD₃ status or sex or age on oral dasatinib PK (**Figure 3-11**, *p<0.05). However, in males at both 8 and 10-weeks age at the 0.25 hr time point, VD₃ deficient mice had significantly higher plasma dasatinib concentrations than VD₃ sufficient mice (**Figure 3-11A,C**).

The oral dasatinib plasma PK in the studied mice was best described using a linear, two-compartment, zero order absorption model, with proportional residual error. Inter-individual variability upon apparent oral systemic clearance (Cl) and apparent volume of distribution of the central compartment (V1) was supported, improved the model fit and performance, as did the off-diagonal correlation between these two parameters. This was defined as the Base model. Inter-occasion variability on either Cl or V1 was not supported, resulted in model instability (failure of FIM convergence via SAEM) and a poorer model fit. This prevented formal testing of age as a covariate but implies that dasatinib PK is not significantly different between 8 and 10-week-old mice. Vitamin D sufficiency and sex were then tested alone and combined as covariates on Cl and were also found to be insignificant at the predefined p<0.05 threshold level. Overall, the Base model without any covariates performed the best, and suggested no influence of vitamin D status, sex, or age on oral dasatinib PK in mice.

Effect of Vitamin D Status on Anti-leukemic Efficacy of Dexamethasone

We recently found that VD₃ deficient mice survived longer with BCR-ABL Arf^{-/-} ALL than sufficient mice in the absence of chemotherapy. We now compared survival from BCR-ABL Arf^{-/-} ALL between VD₃-sufficient and -deficient mice with the oral discontinuous DEX regimen. Leukemia was detected starting at day 19 in multiple VD₃ sufficient mice but only in a few VD₃ deficient mice (**Figure 3-12**). VD₃ sufficient mice began to succumb to tumor burden on day 26. VD₃ deficient mice with DEX treatment survived significantly (**Figure 3-13**, *p < 0.05) longer (median survival time 59 days) compared to the DEX-treated VD₃ sufficient mice (median survival time 36 days). The proportion of total mice surviving BCR-ABL ALL at various time points (**Table 3-3**) further showed that even with DEX therapy VD₃ sufficient mice had poorer survival than

Figure 3-11. Plasma dasatinib pharmacokinetics in VD₃-sufficient and -deficient mice after a single oral gavage of 10 mg/kg dasatinib.

Notes: Plasma dasatinib concentrations in VD₃-sufficient and -deficient 8-week (A) male and (B) female mice, and 10-week (C) male and (D) female mice. A t-test was used to determine the significance of differences between VD₃-sufficient and -deficient mice.

Figure 3-12. Xenogen images tracking disease burden in VD₃-sufficient and -deficient male mice injected with BCR-ABL ALL.

Figure 3-13. Kaplan-Meier curves for the effect of VD₃ sufficiency vs. VD₃ deficiency on survival of mice from BCR-ABL Arf^{-/-} leukemia.

Notes: Kaplan Meier survival curve plotted for male VD₃ sufficient and VD₃ deficient mice with BCR-ABL Arf^{-/-} leukemia and treated with a discontinuous dexamethasone regimen. Prism (version 8) was used to plot and run statistical analysis. The Gehan-Breslow-Wilcoxon test was used to find differences between survival curves of VD₃-sufficient and -deficient groups (*p<0.05)

Table 3-3. Survival proportions of VD₃-sufficient and -deficient mice treated with dexamethasone during survival study from BCR-ABL.

Days survived	VD₃ sufficient	VD₃ deficient
0	100.00	100.00
4	91.67	95.45
15	83.33	
22	79.17	86.36
23	75.00	
24		81.82
25	66.67	
32	58.33	77.27
34	54.17	
35	50.00	
36		72.73
37	41.67	
38	37.50	68.18
46	29.17	
48	25.00	59.09
55	20.83	54.55
59	16.67	45.45

VD₃ deficient mice. In sum, even with DEX chemotherapy, VD₃ sufficient mice have a worse survival outcome than VD₃ deficient mice from BCR-ABL ALL (**Figure 3-13**). No significant differences in serum chemistry except for lower high-density lipoprotein (HDL) levels in VD₃ deficient mice was observed (**Figure 3-14**).

Discussion

There are at least two clinical trials (Dana Farber and Children's Hospital of LA) (ClinicalTrials.gov Identifier number: NCT01574274 and NCT01317940, respectively) administering vitamin D to insufficient and deficient pediatric acute lymphoblastic leukemia patients to restore VD₃ sufficiency. Since the biologically active metabolite of VD₃ (1,25(OH)₂VD₃) induces intestinal CYP3A4 in humans and Cyp3a in mice, and since VD₃ supplementation can alter the pharmacokinetics of orally administered CYP3A4 substrates such as atorvastatin (Schwartz, 2009), we hypothesized that Cyp3a might be higher in the intestine of VD₃ sufficient versus VD₃ deficient mice and that could lead to increased metabolism, decreased systemic exposure, and decreased anti-leukemic efficacy of the orally administered chemotherapies. Because DEX and dasatinib are majorly metabolized by CYP3A4 in humans (Christopher et al., 2008) (Gentile, 1996) and are orally administered to patients orally during ALL therapy (Mitchell et al., 2005) (Foa et al., 2011), this study sought to determine whether there might be unanticipated vitamin D₃ interactions with these orally administered CYP3A metabolized ALL therapies.

In the C57BL/6J mouse model, the effect of VD₃ levels was intestinal specific - VD₃ sufficient female mice had higher levels of intestinal Cyp3a11 compared to VD₃ deficient mice while hepatic Cyp3a11 remained unchanged. This result is relevant to humans because hCYP3A4 was also significantly lower in the intestine of the VD₃ deficient humanized hCYP3A4-transgenic mouse. Consistent with lower intestinal Cyp3a in VD₃ deficient female mice we found serum concentrations of DEX administered by a discontinuous oral regimen were transiently higher at the earliest, but not later times of DEX therapy in these deficient mice. In contrast, when DEX was administered orally as a single oral bolus dose, there was no significant difference in the DEX AUC between the VD₃ sufficient and deficient mice. It is possible that there was no difference in DEX AUC following the single oral DEX gavage because the gut concentration (C_{gut}) achieved ~200 µM (2 mg/kg into a gastric volume of 300 - 400 µL) might saturate Cyp3a (assuming mouse Cyp3a is similar to CYP3A4's K_m of ~25 µM for 6β- and 6α-hydroxydexamethasone formation) (Gentile, 1996). In contrast, mouse intestinal Cyp3a would readily metabolize DEX from the drinking water assuming the DEX C_{gut} is ~7.6 – 10.1 µM at any time-point (assume drinking ~200 - 400 µl / hr). Similar to the findings with dexamethasone, there was no influence of VD₃ status on oral dasatinib PK.

While we chose to test the effect of VD₃ levels on dexamethasone systemic exposure because it is an orally administered anti-leukemic therapy, we would not *a priori* have predicted that intestinal dexamethasone CYP3A-mediated metabolism would be affected by VD₃ levels for several reasons (**Figure 3-15**). First, DEX has a

Figure 3-14. Serum chemistry of VD₃-sufficient and -deficient mice.

Figure 3-15. Cartoon depicting dexamethasone and dasatinib metabolism and efflux transport in intestine and liver.

relatively high oral bioavailability in health individuals (76% and 81% (Loew, Schuster, & Graul, 1986)). Hence, even though DEX can be metabolized by intestinal tissue (Siebe et al., 1993), CYP3A-mediated DEX first-pass metabolism in the gut is probably minimal. Second, the magnitude of DEX drug-drug interactions with CYP3A inducers and inhibitors (low-moderate) supports this conclusion. Third, DEX is known to be primarily cleared by hepatic pathways (Gentile, 1996) undergoing extensive CYP3A4 dependent hepatic metabolism in vivo and in vitro (Tomlinson et al., 1997), and there was no effect of VD₃ status on mouse hepatic Cyp3a5 consistent with the low levels of VDR in hepatocytes compared to intestine (Gascon-Barre et al., 2003). Hence, it was not clear that vitamin D induced variation in intestinal CYP3A4 expression would impact oral DEX systemic exposure.

We chose to test the effect of VD₃ levels on dasatinib pharmacokinetics because this CYP3A substrate is given orally throughout therapy for BCR-ABL ALL. We would have predicted that dasatinib oral bioavailability might be affected by VD₃ levels (**Figure 3-15**). Dasatinib is rapidly absorbed from the gastrointestinal tract after oral dosing, with the C_{max} reached within 0.6 to 2 h. Bioavailability of dasatinib is quite variable with one study showing 45-51% bioavailability (Luo et al., 2006) and other indicating 17% in mice (Kamath, Wang, Lee, & Marathe, 2008). Using bile duct cannulated rats, the percentage of unchanged drug in the gastrointestinal tract was 33% suggesting 67% was absorbed. However, the oral bioavailability in these rats was 27%, which means 63% of drug did not reach the systemic circulation, possibly due to pre-systemic metabolism. Moreover, at least 40% of the total drug (or 60% of absorbed drug) might undergo hepatic metabolism. Dasatinib is primarily metabolized by CYP3A4. How much does intestinal CYP3A4 contribute to dasatinib metabolism? On the one hand co-administration of dasatinib with grapefruit juice is contraindicated (Squibb, 2006) suggesting there is intestinal CYP3A4 metabolism. Moreover, the magnitude of reported interaction with CYP3A inducers and inhibitors (large) supports the assumption of first pass effect at both intestine and liver. However, it should also be considered that CYP3A4-mediated dasatinib metabolism generates CYP3A4 mechanism-based inactivators with a K_i = 6.3 μM and K_{inact} = 0.034 min⁻¹ (X. Li et al., 2009). In our study dasatinib was dosed at 10 mg/kg, the calculated C_{gut} is 1.5 mM, and the C_{max} achieved was 2 μM (0.002 mM). Hence, it is possible that intestinal CYP3A4 undergoes mechanism-based inhibition by CYP3A4-generated metabolites while hepatic CYP3A4 might not.

Is there an intestinal drug efflux transporter-mediated Vitamin D₃ interaction with either dexamethasone or dasatinib? An additional variable affecting oral dexamethasone intestinal absorption could be intestinal Pgp (Schinkel et al., 1995). We first demonstrated that intestinal P-gp is highly inducible not only by drugs (Schuetz, Wrighton, Safe, & Guzelian, 1986) but also by vitamin D exposure (Thummel et al., 2001). Hence, in theory variable VD₃ levels could influence intestinal Pgp expression and contribute to variable DEX absorption and systemic exposure. Dasatinib is also a substrate of Mdr1a and Bcrp that are present at significant levels on the apical membrane of intestinal enterocytes and each has been shown to contribute to limiting dasatinib's oral bioavailability (Lagas et al., 2009) (Chen et al., 2009). Conversely, evidence that

there was a significantly lower dasatinib AUC in Mrp4 KO versus WT mice supports Mrp4 enhanced dasatinib gastric absorption of (Furmanski et al., 2013). However, only Mdr1a has been shown to be induced in enterocytes treated with vitamin D (Thummel et al., 2001). However, not only did our study find no effect of VD₃ levels in mice on dasatinib's AUC, nor any chronic effect on DEX systemic exposure, in fact the expression of Mdr1a mRNA was actually higher in the intestines of VD₃ deficient versus sufficient mice, a paradoxical finding that others have also reported (Quach et al., 2018).

It is also possible that the paradoxical decrease in intestinal drug efflux transporters for dexamethasone (Pgp, Bcrp) and dasatinib (Pgp) in VD₃ Sufficient mice (Figure 3-15) negated the increased intestinal Cyp3a in these same mice. VD₃ deficient mice had significantly lower intestinal Cyp3a expression, but significantly higher Mdr1a levels. The combined opposing effects of metabolism and efflux transport on dexamethasone and dasatinib in this model may have ultimately erased any individual effect of VD₃ on their metabolism or transport.

Why was DEX apparently more rapidly metabolized in VD₃ deficient versus sufficient male only at only the earliest measured time-point in mice given an oral discontinuous dexamethasone regimen? We hypothesized that because DEX over time is inducing intestinal Cyp3a only in the VD₃ deficient mice this is negating the difference in intestinal Cyp3a expression seen between VD₃ deficient versus sufficient mice. The inability to induce Cyp3a in VD₃ in sufficient mice was not because some theoretical maximum intestinal Cyp3a expression level has already been achieved in VD₃ sufficient mice because the threshold cycle (Ct value) for Cyp3a amplification was lower in VD₃ deficient mice treated with DEX than in vitamin D sufficient mice with or without DEX (i.e., the relative expression level of Cyp3a in the DEX induced VD₃ deficient mice was higher than the Cyp3a level in sufficient mice). Indeed, the selective DEX induction of intestinal Cyp3a in VD₃ deficient mice, but not sufficient mice (**Figure 3-10**), suggests there is competition of VDR and PXR at the VDRE/PXRE binding sites in the Cyp3a promoter. VDR/NR1I1 and PXR/NR1I2, both members of the NR1I family of nuclear hormone receptors, share common DNA binding elements (Reschly & Krasowski, 2006). We previously showed that VDR-RXR and PXR-RXR heterodimers bind to the identical DNA elements in the Cyp3a promoter (Thummel et al., 2001). Moreover, there is precedence for VDR-RXR and PXR-RXR heterodimers competing at common binding elements because PXR bound by the potent ligand rifampin was previously shown to inhibit VD₃-ligand activated VDR binding to the CYP24 promoter in intestinal cells (C. Zhou et al., 2006). Results from this study suggest that in sufficient mice VD₃ activated VDR-RXR bound to the Cyp3a VDRE/PXRE is preventing DEX (a relatively weak PXR agonist (K_d~30 µM))-activated PXR-RXR from displacing VDR from the VDRE/PXRE binding site (**Figure 3-16**). Conversely, in VD₃ deficient mice the VDRE/PXRE is unoccupied by VDR because the VD₃ ligand level is insufficient to ligand activate DNA binding, so the DEX -activated PXR-RXR can readily bind to and activate the Cyp3a promoter (**Figure 3-16**).

Tissue-specific DEX induction of Cyp3a in intestine but not liver, but DEX induction of Cyp2b in liver, suggests compartment specific induction of these Cyps

Figure 3-16. Cartoon depicting hypothesis that dexamethasone-activated PXR can induce Cyp3a in VD₃ deficient mice, but not in VD₃-sufficient mice where 1,25(OH)₂D₃-activated VDR blocks DEX-PXR induction.

by PXR and the glucocorticoid receptor (**Figure 3-17**). The dose response curves for glucocorticoid activation of GR versus PXR are strikingly different (**Figure 3-17**). GR is ligand activated at nM concentration of glucocorticoids and makes physiological sense as these steroids need to be maintained within a strict homeostatic range. In contrast, the ED50 for PXR activation by glucocorticoids is approximately 30 μ M (Kliewer et al., 1998). Indeed, PXR requires stress/pathological levels and higher levels of glucocorticoids to be activated and then to induce Cyp3a and other detoxification pathways. If PXR were activated by glucocorticoids at nanomolar concentrations this would profoundly perturb endogenous glucocorticoid homeostasis. In contrast, Cyp2b is transcriptionally activated by the glucocorticoid receptor (GR) and we previously showed GR is required for DEX induction of Cyp2b in mice (Schuetz et al., 2000). Conversely, glucocorticoid induction of Cyp3a occurs via PXR with no participation of, or requirement for, GR (Schuetz et al., 2000). The observed systemic concentrations of DEX (2.6 – 201.5 nM) in mice on the discontinuous regimen were in the nanomolar range which would activate GR, but not PXR, in liver. Accordingly, only Cyp2b was induced by DEX -activated GR in liver (**Figure 3-17**). However, the gut concentration of orally administered DEX would readily activate intestinal PXR. Importantly, these results are directly relevant to the pediatric ALL patients treated with DEX as the plasma concentrations achieved with the oral discontinuous regimen in mice are similar to those achieved in patients treated with conventional DEX therapy (Ramsey et al., 2014).

We recently found in a murine BCR-ABL ALL model VD₃ sufficient mice died earlier than VD₃ deficient mice (Chapter 4). Given our finding that VD₃ deficient mice had higher systemic DEX exposure compared to sufficient mice during the first days of discontinuous DEX treatment we might have expected DEX treatment to negate this VD₃ deficient survival advantage. However, DEX treatment was unable to reverse the survival advantage of VD₃ deficient over deficient mice with ALL. It remains to be determined whether the effects of VD₃ on survival of mice from BCR-ABL ALL translate to humans.

The 5-year event free survival rates for pediatric ALL have almost reached 90% (Strahlendorf, 2018). However, it is always important to consider whether a therapeutic intervention –e.g., vitamin D₃ to treat vitamin D deficiency, might cause an unexpected negative side-effect – in this case an unfavorable CYP3A vitamin D₃-mediated drug interaction. Our results in mice suggest that vitamin D supplementation to vitamin D insufficient/deficient patients will not affect systemic exposures of DEX or dasatinib. Our results do suggest the intriguing possibility that whether a patient is VD₃ sufficient versus deficient may have an impact on the magnitude of CYP3A-mediated drug interaction with CYP3A inducers (including DEX) for substrates that undergo significant first pass intestinal metabolism (e.g., atorvastatin) (Schwartz, 2009).

Figure 3-17. Differences in plasma and gut dexamethasone concentrations following oral discontinuous regimen leads to tissue specific activation of GR and PXR.

Data source: Kliewer, S. A., Moore, J. T., Wade, L., Staudinger, J. L., Watson, M. A., Jones, S. A., . . . Lehmann, J. M. (1998). An orphan nuclear receptor

CHAPTER 4. ROLE OF VITAMIN D IN BCR-ABL ARF-/- ACUTE LYMPHOBLASTIC LEUKEMIA¹

Introduction

Vitamin D deficiency affects an estimated 1 billion people in the world across all ethnicities and age groups (Boscoe & Schymura, 2006; Holick, 2007; Holick et al., 2011). VDD is an independent risk factor for mortality in the general population (Melamed, Michos, Post, & Astor, 2008) and almost 60% of children with malignant diseases have suboptimal vitamin D levels (Sinha, Avery, Turner, Bailey, & Cheetham, 2011). VDD is not limited to developing countries, a recent study found that among 45 people tested in Memphis, TN for vitamin A and vitamin D levels, only two individuals had sufficient levels of both vitamins (B. G. Jones et al., 2016).

Vitamin D is a fat-soluble vitamin that not only regulates calcium absorption and bone metabolism, but can also regulate cell proliferation, differentiation and the immune response. The biologically active form of Vitamin D, 1, 25(OH)₂VD₃, binds to the vitamin D receptor (VDR) that heterodimerizes with the retinoid X receptor (RXR), and that complex binds to VDR-RXR response elements in target genes to regulate transcription. VDR is highly expressed in intestine, kidney and bone, but also in normal and neoplastic hematopoietic cells and mesenchymal stem cells in bone marrow (Haussler et al., 1998). 1, 25-(OH)₂VD₃ can modify embryonic hematopoietic stem and progenitor cell production (Cortes et al., 2016). 1, 25(OH)₂VD₃ inhibits proliferation of mouse and human myeloid leukemia cells (Tanaka et al., 1982) and stimulates myeloid cell differentiation into mature macrophages. Indeed, mice with acute myeloid leukemia when treated with analogs of 1, 25(OH)₂VD₃ survived longer than the untreated mice (Abe et al., 1981). Moreover, vitamin D insufficiency/deficiency is associated with poor clinical outcome and significantly worse progression free survival of patients from AML (H. J. Lee et al., 2014), chronic lymphoid leukemia (Shanafelt et al., 2011), and non-Hodgkin lymphoma (Drake et al., 2010) indicating the clinical significance of vitamin D levels in patients with AML and CLL.

Acute lymphoblastic leukemia (ALL) is the most common form of childhood cancer accounting for almost 80% of pediatric leukemias (Madhusoodhan, Carroll, & Bhatla, 2016), and multiple reports suggest that a majority of leukemia patients are VD₃ deficient at the time of diagnosis (Naz, Qureshi, Shamsi, & Mahboob, 2013; Sinha et al., 2011). Accordingly, both Dana Farber (Identifier number: NCT01574274) and Children's Hospital of LA (Identifier number: NCT01317940) have ongoing pediatric ALL clinical trials to monitor patients' VD₃ levels and to give supplementation to restore sufficiency. However, it is not known how VD₃ deficiency or supplementation might affect ALL

¹ Modified from final submission with open access permission. Annu, K., Cline, C., Yasuda, K., Ganguly, S., Pesch, A., Cooper, B., . . . Schuetz, E. G. (2020). Role of Vitamins A and D in BCR-ABL Arf(-/-) Acute Lymphoblastic Leukemia. *Sci Rep*, 10(1), 2359. [doi:10.1038/s41598-020-59101-4](https://doi.org/10.1038/s41598-020-59101-4) (*Co-first author) (Annu et al., 2020).

progression or survival. Among the few reports of VD₃ effects on the B-cell lineage of ALL (B-ALL) (the most common form of pediatric ALL), there have been conflicting results; one study concluded that VD₃ had no effect on leukemia cell growth in suspension cultures (Antony et al., 2012), while another study concluded that VD₃ inhibited B-cell ALL growth in a clonogenic soft agar assay (Consolini et al., 2001). Given the controversy in the literature, and the fact that a considerable number of ALL patients are VD₃ deficient and some of them are receiving VD₃ supplementation, it is important to test whether VD₃ levels affect B-cell ALL cell growth, survival and prognosis.

B-ALL is classified into different sub-types based on a number of chromosomal abnormalities, and loss-of function or dominant-negative sequence mutations. Three-to-five percent of pediatric ALL cases and 25% of adult ALL cases, carry the translocation between chromosomes 9 and 22 [t(9;22)] creating the BCR-ABL1 fusion gene (the Philadelphia chromosome (Ph+)) (Williams & Sherr, 2008). At diagnosis around 67% of pediatric Ph+ ALL patients also have Ink4-Arf deletion (Arf-/-) (hereafter called BCR-ABL ALL or BCR-ABL+ ALL) and approximately 80% have IKAROS (IKZF1) alterations, which are considered hallmarks for high-risk B-ALL (Mullighan et al., 2008). Therefore, we have tested the effects of vitamin D sufficiency versus deficiency on survival in a murine BCR-ABL leukemia model and probed the mechanisms of their differential effects.

Materials and Methods

Animals

C57BL/6, day 4-5 estrus pregnant (E4-5) female mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Animals arriving at the St. Jude Children's Research Hospital (SJCRH) animal facility were placed Purina Mills (Purina Mills, St. Louis, MO, USA) diets – either control (cat. no. 5W9M contained 1.5 IU of vitamin D), VDD (cat. no. 5W9X, contained 0 IU of vitamin D) were used for the survival and other *in vivo* studies.

Pregnant dams were maintained on these diets from the time of arrival, throughout the pregnancy, and after parturition. At weaning mouse pups were maintained on their respective diets. We also used a different strategy to generate vitamin D deficiency in mice by transitioning them to a VDD diet at weaning. This would eliminate any potential effect of a vitamin D deficient diet on the status of any other systems in pups derived from pregnant mom's put on VDD diets at pregnancy day 5. Hence, mice in a separate set of survival studies, were placed at weaning on a VDD diet (Harlan catalog no. 5A69, 0 IU of vitamin D) and vitamin D sufficient control diet (catalog. no. 5BV8, 3.3 IU of vitamin D).

Serum 25-(OH) VD₃ levels were routinely measured from representative mice in the VDD group (starting at 4 weeks of age) to determine vitamin D status. A level of 20-50 ng/mL is considered vitamin D sufficient; 12-20 ng/mL is vitamin D insufficient, and less than 12 ng/mL is vitamin D deficient. Sera were sent to Michigan State University (Lansing, MI, USA) for 25-(OH) VD₃ testing. Serum 25-(OH) VD₃ levels were also measured in mice at the terminal time point in the leukemia survival studies.

Ethical Approval Statement

Association for Assessment and Accreditation for Laboratory Animal Care (AALAC) guidelines and Institutional Animal Care and Use Committee (IACUC) approved protocols were followed for all the experimental procedures and housing of the mice. All methods were carried out in accordance with the relevant guidelines and regulations.

Leukemia and Bone Marrow Stroma Cells

Murine BCR-ABL⁺ Arf^{-/-} cells tagged with luciferase (Luc⁺) or green fluorescent protein (GFP⁺) (Williams, Roussel, & Sherr, 2006) created by Dr. Charles Sherr's lab and provided by Dr. Mary Relling's lab, were cultured as previously described (Ramsey et al., 2015). Human mesenchymal stromal cells (hMSCs, bone marrow stroma) immortalized with telomerase (Mihara et al., 2003) and created by Dr. Dario Campana's lab, were provided by Dr. Jun Yang's lab, and cultured as described (Iwamoto, Mihara, Downing, Pui, & Campana, 2007).

BCR-ABL ALL Disease Generation and Monitoring

On day zero, 2,000 BCR-ABL Arf^{-/-} Luc⁺ cells were injected retro-orbitally into syngeneic, unconditioned 6-10-week-old mice, alternating injection order between cages and groups. The first experiment was run with control male (M) (n=17), VDD M (n=23) and control female (F) (n=14), VDD F (n=14) mice. Mice were anesthetized with isoflurane and images using Xenogen IVIS-200 (Caliper Life Sciences, Hopkinton, MA) were acquired post-intraperitoneal injection of 200 µL of 100mg/kg D-Luciferin (Caliper Life Sciences, Hopkinton, MA). The bioluminescent signal (photons/s) was acquired from a fixed region of interest (ROI) starting on day 8, repeating every 3-4 days to monitor disease progression (**Figure 4-1**). Acquired images were analyzed with Living Image 3.1 software (Caliper Life Sciences). BCR-ABL-Luciferase disease burden was quantified from whole animal total luciferase flux measurements (photons/s) from the images.

Moribund state of mice was determined based on hind limb paralysis, a scruffy coat, lethargy, or an inability to obtain food or water. At this time before euthanasia, blood was collected for the analysis of cholesterol, triglycerides, high-density lipoprotein

Figure 4-1. BCR-ABL ALL disease generation and monitoring in mice.

(HDL) and low-density lipoprotein (LDL), white blood cell number, and serum for 25-(OH) VD₃. Spleen was harvested and bone marrow cells from femur were flushed using a needle with either cell culture media or RNeasy lysis buffer (Qiagen, Gaithersburg, MD, USA). The cells were transferred to a 1.5 mL eppendorf tube and pelleted, the cell pellets were resuspended in 100 µL PBS, and transferred to white, clear-bottomed 96-well plate (Costar, Corning Inc., Kennebunk, ME, USA) to which 100 µL of Bright-Glo™ (Promega, Madison, WI, USA) was added. A standard curve with a range of 3,000 to 2 million BCR-ABL cells was used in order to convert the luminescence value into the number of BCR-ABL cells per well. Luminescence was measured using the BioTek Synergy™ 4 Hybrid Microplate Reader.

A portion of spleen, hind limb was individually fixed in 10% formalin; 24 hours later the tissues were transferred to Cal-Rite (Thermo Scientific, Pittsburgh, PA) for a minimum of 48 hours. Tissues were embedded in paraffin, and 4 µm sections were cut and stained with hematoxylin and eosin. Slides were evaluated by a pathologist to determine the extent of leukemia burden, scoring the tissues on a 6- point scale from 0-5. The entire experiment was repeated, and both studies were combined for the data analysis with a total of control M (n=34), VDD M (n=34) and control F (n=27), VDD F (n=25) mice.

To measure bone marrow vascularity and trabecular volume, the slides were scanned using an Aperio ScanScope (Aperio Technologies, Vista, CA) with a 20X objective. Measurements were performed using ImageScope software. The metaphyseal region defined as beginning at the physis and ending 1mm distal to it; this marrow area was outlined to obtain total metaphyseal area. For measurement of bone marrow vascularity all vascular lumens (sinusoids) were outlined within that area. Two types of data calculated from these measurements, % vascular area (µm² vessel lumen/ µm² marrow), and length (µm vessel/ µm² marrow). For trabecular volume, all trabeculae were outlined within this area and the output was calculated as % trabecular area (µm² trabecular bone/ µm² marrow). Osteoblast and osteoclast numbers, as well as leukemia burden, were evaluated semi-quantitatively by a veterinary pathologist (L.J.J.) using a 5-point scale.

PCR Assays

PCR to determine gender of BCR-ABL ALL cells

The gender of the murine BCR-ABL+ Arf^{-/-} cells was determined by PCR of genomic DNA for the presence of X and Y-chromosome specific markers (McFarlane, Truong, Palmer, & Wilhelm, 2013). DNA was amplified with the following primer pairs:

Sly/Xlr_F, 5'-GATGATTTGAGTGGAAATGTGAGGTA-3';
Sly/Xlr-R, 5'-CTTATGTTTATAGGCATGCACCATGTA-3' and
Zfy_F, 5'-GACT A GACATGTCTTAACATCTGTCC-3' and

Zfy_R, 5'-CC T A TTGCATGGACTGCAGCTTATG-3' and amplification conditions were as described (McFarlane et al., 2013).

PCR to detect VDR mRNA

The expression of VDR in BCR-ABL+ Arf-/- and LS180 cells was determined by PCR. DNA was amplified using the following primer pairs:

mVDR-F, CTCCTCGATGCCCACCACAAGACCTACG;
mVDR-R, GTGGGGCAGCATGGAGAGCGGAGACAG;
hVDR-F, CGGCCGGACCAGAAGCCTTT;
hVDR-R, CGGGGCACGTTCCGGTCAAA;
GAPDH-F, ACCACAGTCCATGCCATCAC;
GAPDH-R, TCCACCACCCTGTTGCTGTA.

Promega PCR master mix (catalog number M7505), and amplification conditions are as described below: Denaturation at 95°C for 2 min; 34 cycles (mVDR) or 40 cycles (hVDR): denaturation at 95°C for 30sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec; extension at 72°C for 5 min; 4°C hold. The product was mixed with 6x DNA loading dye (Promega, WI, USA) and ran on a 2% agarose gel (used SYBR Safe DNA gel stain) at 100 V for 90 minutes. Images were taken using Image Lab Software™.

Analysis of *in vivo* Growth of Leukemic Burden

Change in leukemia burden with time was analyzed from the change in whole body luminescence data. Luminescence data for every mouse was normalized with its baseline luminescence value from day 8. Fold change in luminescence over baseline was plotted against days, which was assumed to follow the exponential growth equation, $\text{Fold change in luminescence} = A e^{(B \cdot t)}$, where, A is the intercept and B is the slope of the exponential growth curve. Parameters A and B were estimated by fitting the fold change in luminescence versus days for each mouse using Microsoft Excel-based trendline analysis. Correlations were considered acceptable only if $r^2 > 0.7$. All the survivors were removed from the analysis, as we do not expect to have increase/growth in leukemia burden in these mice. Other filtration criteria were: 1) fold change in luminescence has to be > 1 , i.e., increase in luminescence over the baseline, 2) positive correlation of fold-change in luminescence with days, 3) there have to be at least three data points for the assessment of correlation, and 4) since the same number of leukemic cells were injected into the mouse, we expected to have no significant difference in intercept A and thus outlier intercepts were removed from the analysis. After filtration of the data, individual slopes (B) were computed for each mouse and compared between the groups, data represented as median slope $\pm$ range and Mann-Whitney test at $p < 0.05$ was used to analyze the significance.

BCR-ABL ALL Viable Cell Number Assay

Luciferase-tagged BCR-ABL cells were plated at a density of 500 cells per well on a 24 well plate and treated with either 1,25-(OH)₂VD₃ (50 or 100 nM) or ethanol (vehicle) and the plates incubated at 37°C 8% CO₂. To select the concentration of 1,25-(OH)₂VD₃ in cell culture, we first measured dose response effect on BCR-ABL ALL viable cell number and found no effect with 1-10 nM, but equal effectiveness of 50-100 nM. Since the duration of our 1,25 (OH)₂VD₃ studies was routinely 48 hr (but as long as 96 hr), and the vitamin is not replenished after dosing at zero hr, and 1, 25-(OH)₂VD₃ has a half-life of 15 hr, the effective concentration remaining at the end of a 48 hr experiment following a 100 nM treatment is 25 nM.

In humans the bone marrow concentration of 1, 25-(OH)₂VD₃ is 500-fold higher than the bone marrow plasma concentration (Blazsek et al., 1996). Since the plasma concentration of 1,25(OH)₂VD₃ is 30 pg/ml (0.072 nM), the bone marrow stroma concentration at 500-times that = 36 nM. Hence, 50-100 nM 1,25(OH)₂VD₃ was used. To measure cell number, at the terminal time point for vehicle, 1,25(OH)₂VD₃ treated wells, the entire contents of each well were transferred to a 1.5 mL eppendorf tube, the cells were pelleted, resuspended in 100µL PBS, and transferred to white clear-bottomed 96-well plates (Costar Corning Inc., Kennebunk, ME, USA) and 100µL of Bright-Glo™ (Promega, Madison, WI, USA) was added. Luminescence was read using the BioTek Synergy™ 4 Hybrid Microplate Reader. Standard curves were prepared in triplicate using known numbers of Luc+ BCR-ABL cells, then the number of BCR-ABL cells in experimental samples was determined by interpolation, and results plotted of average cell number/treatment group or fold differences between vitamin treated and vehicle-treated wells. Hence, the assay measures the difference in total number of cells between treatment groups at a single time point. The assay measures number of viable (not dead cells) because the assay depends on the number of cells that are viable and metabolically active.

In vitro Homing Assay

hMSC (100,000/well) were plated onto the bottom of 24-well plates and treated with ethanol vehicle or 100 nM 1,25(OH)₂VD₃ for 48 hours with half of the wells/group treated with media lacking fetal bovine serum (FBS). Identical 24-well plates without hMSC were prepared. Then 6.5mm Transwell Permeable Supports with a 3 µm polycarbonate membrane (Costar Corning Inc., Kennebunk, ME, USA) were added to each well and 1 million BCR-ABL luciferase(luc)+ cells in 100 µL of media was added to top donor compartment and incubated at 8% CO₂ for 24 hr. The bottom of each transwell membrane (**Figure 4-2**) was then washed with media to collect any attached cells. A cell scraper was used to remove all cells from the wells, which were pelleted and resuspended in 100 µL PBS and 100 µL of Bright-Glo™ (Promega, Madison, WI).

The BioTek Synergy™ 4 Hybrid Microplate Reader was used to read the luminescence on a white, clear-bottom 96 well plate (Costar, Corning Incorporated,

Figure 4-2. BCR-ABL cell migration in vitro using transwell membrane.

Kennebunk, ME, USA). Luminescence from 3,000 - 2 million BCR-ABL luc+ cells was used to generate a standard curve and the relative BCR-ABL cell number in the receiver compartment was interpolated from the standard curve which was used to determine the percentage of total cells that had migrated from the donor to the receiver compartment.

Real-Time PCR

A confluent layer of human bone marrow mesenchymal stem cells (hMSCs) immortalized with telomerase (Mihara et al., 2003) were treated with vehicle or 100nM 1,25(OH)₂VD₃ for 48 or 72 hours. RNA was extracted using Trizol (Ambion Life technologies, CA, USA or Thermo Fisher Scientific, CA, USA) and the Qiagen RNEasy clean-up kit (Hilden, Germany). 500ng of RNA was used to create cDNA using the ThermoScript™ RT-PCR System (Invitrogen ThermoFischer, CA, USA) that was then diluted to a total volume of 60μL. Primers (Invitrogen, Carlsbad, CA) for amplification of human CXCL12/SDF-1α were (F) 5'-AGAACTGTTGGCAAGGTGACA-3' and (R) 5'-CTGACATTCATATGGCTCTCATTC-3'; and for mouse CXCL12/ SDF-1α were (F) 5'-CAGTGACGGTAAACCAGTCAGC- 3' and (R) 5'-TGGCGATGTGGCTCTCG- 3'. Samples were run at 95° for 15 minutes and then 40 cycles of 92° for 30 seconds, 60° for 30 seconds, and 68° for 1 minute followed by a dissociation step for the real-time PCR.

CXCL12 ELISA Assay

The Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) for human CXCL12/SDF-1α was used to assay both cell culture media and mouse serum according to the manufacturer's protocol. A standard curve was generated using the included controls and used for comparison to a positive control as well as the unknown samples. Cell culture media samples (n=3) from hMSCs in a 6-well dish were assayed undiluted after 48-hour treatment with either ethanol or 100nM 1,25(OH)₂VD₃. Undiluted serums from mice on either a VD₃ sufficient (control) or VD₃ deficient diet (VDD group; n=5 per group) were also assayed using this approach. The BioTek Synergy™ 4 Hybrid Microplate Reader was used to measure fluorescence at 450 nm and 540 nm. Final values used for comparison were determined by subtracting the reading at 540 nm from the reading at 450 nm; this step was designed to correct for optical imperfections in the plate.

Immunohistochemistry

VDD and control mice were perfused with 4% paraformaldehyde and the hind limbs were fixed in 10% neutral buffered formalin, decalcified, embedded in paraffin, sectioned (4 μm) and placed onto glass slides for staining. Antibodies for IHC were obtained from Abcam and diluted in Phosphate Buffer Saline with 0.3% Triton X-100 (PBST). Slides containing either mouse tibia or mouse hind limb were deparaffinized and antigens were retrieved using Target Retrieval solution pH 6.0 (Dako, Carpinteria CA, USA) in a pressure cooker for 15 min. After retrieval, slides were rinsed with PBST,

treated with 3% hydrogen peroxide, and blocked with Background Sniper (Biocare Medical, Pacheco, CA, USA). Slides were incubated overnight at 4°C with primary specific antibodies (rabbit anti-collagen I, ab34710 (1:500) and rabbit anti-collagen V (ab7046) (1:50), or the appropriate isotype control antibody, rinsed twice with PBST, and incubated with the secondary antibody (Donkey anti-rabbit 568, ab10042) for 2 hr. Slides were rinsed again in PBST and mounted using ProLong® Gold Antifade Mountant with DAPI (Molecular Probes by Life Technologies™, Eugene, OR, USA) and allowed to dry overnight before viewing. Images were obtained (60X magnification) using the Marianas Microscopy System (Intelligent Imaging Innovations).

Flow Cytometry

Age matched VD₃ Sufficient and deficient mice are used for this assay. Isolation of bone cells was performed as described in (Houlihan et al., 2012) with minor modifications to the protocol. Cardiac puncture was performed to harvest blood which is processed into serum and stored at -80°C. This serum is used to measure the 25-(OH) VD₃ levels in the mice. After euthanizing the mouse, both tibia and femur were dissected by remove any adherent muscle tissue and cleaned carefully using Kimwipe to remove any nonadherent tissue (**Figure 4-3**). Using a syringe filled with cold PBS, bone marrow is flushed into a different test tube. The bones are placed in 30 mL of ice-cold PBS. The bones were washed 3 times in fresh PBS by vigorous shaking. Using a sterile mortar and pestle, the bones were crushed with one fracture per bone and using scissors to cut the bone into small fragments. The bone fragments were washed 2-3 times using freshly made 10 mL of HBSS+ solution. HBSS+ is made using Hank's balanced salt solution (HBSS) (Invitrogen, cat. no 14170112) as described in (Houlihan et al., 2012). Then the bone fragments are placed into 50 mL conical tubes filled with 20 mL of preheated DMEM with 0.2% collagenase and placed on a temperature regulated shaker at 37°C for 1 hour at 110 r.p.m. Then, the solution is filtered using a 70 µm cell strainer and placed on ice. The fragments are placed into the mortar with HBSS+ solution and using pestle gentle tapping is performed to liberate the bone cells into the solution. After performing this step at least 6-7 times to obtain good yield, the suspension is centrifuged in a precooled centrifuge at 280 g for 10 min at 4°C. Supernatant was discarded carefully and for red blood lysis to occur, the pellet was resuspended in 1 mL of ice-cold water for 6 sec. Immediately 1 mL of 2xPBS with 4% (v/v) FBS was added to stop the lysis and the volume was made up to 15 mL using HBSS+. The solution is filtered through 70 µm cell strainer and spined once again for 5 minutes at 280 g in 4°C. Supernatant was discarded carefully, and the pellet was resuspended in 1 mL of HBSS+. This was later used for staining of the bone cells. The antibodies used, concentrations and isotypes used to stain the cells are shown below in the **Table 4-1**.

Data Analysis

Kaplan Meier curves were generated to compare survival data between the groups and the Gehan-Breslow-Wilcoxon Test was used to determine significance. The Welch's

Figure 4-3. Experimental design - bone/bone marrow cells isolation and staining

Table 4-1. Antibodies used to stain bone and bone marrow cells for flow cytometry.

Protein and fluorochrome	Company, Cat. no.	Concentration	Isotype
CD31, FITC	Biolegend, 102405	0.5 mg/mL	Rat IgG2a, K
CD51, PE	Biolegend, 104105	0.2 mg/mL	Rat IgG1, K
Sca-1, APC-Cy7	Biolegend, 108125	0.2 mg/mL	Rat IgG2a, K
CD140a, PE-Cy7	Biolegend, 135911	0.2 mg/mL	Rat IgG2a, K
CD140b, APC	ThermoFisher Scientific, 17-1402-82	0.2 mg/mL	Rat IgG2a, K
CD45, BV605	Biolegend, 103139	0.2 mg/mL	Rat IgG2a, K
Ter119, BV711	Biolegend, 116233	0.2 mg/mL	Rat IgG2b, K
CD71, BV786	BD Biosciences,		Rat IgG2b, K

t-test was used to determine significant differences in ROI means between the groups. Luciferase signals of blood, spleen, and bone marrow, and ELISA data were analyzed using Mann-Whitney nonparametric test on GraphPad Prism versions 5 and 7. Blood chemistry data were analyzed using 1way ANOVA with Tukey's multiple comparison test. Significance is calculated at $p < 0.05$ and outliers were determined using Grubb's test on GraphPad QuickCalcs. Real-Time PCR data was analyzed using the $\Delta\Delta C_t$ method. Unpaired t-tests were used to determine significance in other assay's comparing cells treated with $1,25(\text{OH})_2\text{VD}_3$ or vehicle. Flow cytometry comparisons were done using unpaired t-tests using GraphPad Prism software.

Results

Vitamin D Deficiency Improves Survival of Mice with BCR-ABL Arf^{-/-} B-ALL

Progression of BCR-ABL Arf^{-/-} luciferase tagged pre-B leukemia cells and survival from disease were compared between vitamin D sufficient control mice and VDD mice by Xenogen imaging of leukemic cells starting on day 8 in male and female mice (**Figures 4-4, 4-5**). Leukemia was detected at day 8 in control male mice but was not detected in the VDD male mice until day 10. Surprisingly, compared to VD_3 sufficient (control) mice, VDD mice showed a slower tumor progression over time and had lower average disease burden per mouse (**Figure 4-6**). Consequently, VDD mice survived significantly longer (**Figure 4-7**, $**p=0.003$) than VD_3 sufficient control mice. This can also readily be seen by comparing the proportion of mice surviving on each day after leukemia was administered (**Table 4-2**). For example, study 1 on Day 18 only 47% of the control male mice versus 80.95% of the VDD male mice were surviving, hence the median survival for the control male mice was 18 days versus 21 days for the VDD male mice. Female mice inoculated with tumor cells had slow growth of disease and longer median survival than males, so we determined the sex of the murine BCR-ABL leukemia cells using a PCR assay to discriminate X and Y chromosome specific genes in the leukemia cells. This PCR assay relies on Y chromosome specific Zfy amplification in males, but is absent in females, and amplification of a 280 bp Y chromosome specific Sly gene product in males, and a 480/685 bp X chromosome specific Xlr gene product in females (McFarlane et al., 2013). The BCR-ABL cells were male as indicated by the Zfy and Sly Y chromosomal PCR products (**Figure 4-8**).

Vitamin D deficiency was confirmed in serum obtained from terminal blood samples by analysis of 25-(OH)VD_3 in the control, VDD mice (**Table 4-3**). Complete blood count (CBC) (**Table 4-4**) and serum chemistries (**Table 4-5**) were compared between control, VDD mice with BCR-ABL ALL diseased and non-leukemic healthy mice (no disease controls (ND)). Compared to leukemic control mice, leukemic male mice deficient for vitamin D had decreased red blood cell number, hematocrit and hemoglobin consistent with higher leukemic burden in these mice. Comparing serum chemistries between the groups, the vitamin deficient males had elevated levels of HDL

Figure 4-4. BCR-ABL luc⁺ ALL disease burden in male mice.

Figure 4-5. BCR-ABL luc⁺ ALL disease burden in female mice.

Figure 4-6. BCR-ABL ALL disease burden on day 17 in male control and VDD mice.

Notes: The unpaired t-test with Welch's correction was used to compare leukemia body burden (**p=0.001)

Figure 4-7. Kaplan Meier survival curve of male VD₃ sufficient (control) and VD₃ deficient mice.

Notes: The Gehan-Breslow-Wilcoxon test was used to find differences between survival curves of control and each vitamin deficient group (***p<0.000, **p<0.01)

Table 4-2. Survival proportions of control and vitamin D-deficient mice.

Days elapsed	Control	Vitamin D deficient
0	100	100
5		
16		95.24
17		85.71
18	47.06	80.95
19	11.76	71.43
20		61.90
21	0.00	42.86
24		33.33
25		9.52
29		0.00

Notes: Control = Vitamin D sufficient

Figure 4-8. Determination of mouse gender using Sly/Xlr and Zfy PCR.

Notes: Genomic DNA from YY male and XY female mice were amplified with primers for (A) Y chromosome Sly intron 8 and (B) X chromosome Xlr intron 6 and Y chromosome Zfy.

Table 4-3. Serum 25-(OH) VD₃ values across groups (Representative data).

Group	Gender	25-(OH) VD₃ (ng/mL)
CON	F	102.8
CON	F	50.4
CON	M	87.6
CON	M	36.4
VDD	F	2.4
VDD	F	1.6
VDD	M	3.2
VDD	M	2.4

Notes: CON = VD₃ sufficient, VDD = VD₃ deficient, M = Male, F = Female.

Table 4-4. VD₃-sufficient (control), -deficient (VDD) male (M) mice complete blood count (CBC).

Cell type	Control M		VDD M		Control M ND		VDD M ND	
	Average	S.D.	Average	S.D.	Average	S.D.	Average	S.D.
WBC (X 103/uL)	45.94	30.1	38.78	15.48	2.55	2.19	5.57	0.72
Neutrophil (#)	18.46	9.19	17.22	7.55	0.39	0.32	1.11	0.11
Lymphocyte (#)	15.43	13.26	10.78	4.97	1.97	1.71	4	0.73
Monocyte (#)	10.14	8.16	7.07	2.93	0.19	0.17	0.47	0.11
Eosinophil (#)	1.73	1.43	2.84	2.37	0.01	0.01	0.01	0.01
Basophil (#)	0.19	0.21	0.87	0.78	0.01	0.01	0	0
Nucleated RBC (#)	0	0	0	0	0	0	0	0
Neutrophil (%)	41.99	8.07	44.76	7.82	15.22	0.51	19.89	0.62
Lymphocyte (%)	32.74	7.56	28.37	8.85	76.98	0.74	71.46	3.83
Monocyte (%)	21.21	5.42	18.31	2.47	7.1	0.62	8.54	3.09
Eosinophil (%)	3.67	2.78	6.56	3.12	0.45	0.46	0.13	0.11
Basophil (%)	0.38	0.4	2	1.05	0.26	0.37	0	0
Nucleated RBC (%)	0	0	0	0	0	0	0	0
Hematocrit (%)	33.5	2.94	28	4.31	31.6	1.41	31.05	2.19
RBC (X 106/uL)	8.03	0.71	6.63	0.91	7.89	0.45	7.55	0.91
Hemoglobin (g/dL)	12.01	1.18	10.02	1.39	12.05	0.49	11.6	0.71
Mean Corpuscular Volume (fL)	41.72	1.38	42.12	1.02	40.05	0.49	41.25	2.05
Mean Corpuscular Hemoglobin (pg)	14.95	0.58	15.1	0.47	15.3	0.28	15.45	0.92
Mean Corpuscular Hemoglobin Conc (g/dL)	35.83	0.76	35.89	1.32	38.1	0.14	37.35	0.35
Red Cell Distribution Width (%)	13.45	0.91	12.77	0.34	13.7	0.57	13.95	0.49
RSD	5.61	0.56	5.38	0.19	5.5	0.14	5.75	0.49
Reticulocyte (#)	14.69	17.73	4.32	5.03	9.85	12.8	8.95	12.66
Reticulocyte (%)	0.2	0.25	0.07	0.09	0.13	0.17	0.13	0.18
Platelet (X 103/uL)	412.8	114.23	257.33	43.81	505.5	27.58	469.5	154.86
Mean Platelet Volume (fL)	5.68	0.39	5.54	0.35	5	0	4.85	0.07
Platelet Distribution Width	65.15	9.56	66.86	9.29	48.9	0.42	49.8	0.57
Plateletcrit	0.24	0.08	0.14	0.03	0.25	0.01	0.23	0.07

Notes: Control = VD₃ sufficient, VDD = VD₃ deficient, M = Male, ND = No disease, S.D. = Standard deviation.

Red font indicates higher levels compared to control group; Green font indicates lower levels compared to control group

Table 4-5. VD₃-sufficient and -deficient male mice serum chemistry.

Parameter	Control M		VDD M		Control M ND		VDD M ND	
	Average	S.D.	Average	S.D.	Average	S.D.	Average	S.D.
Triglyceride (mg/dl)	52.99	15.89	44.63	13.17	66.15	3.04	39.15	17.61
HDL (mg/dl)	47.24	12.71	58.46	10.49	87.15	6.58	45.25	42.78
LDL (mg/dl)	9.15	1.99	18.31	16.24	8.45	0.49	4.10	3.25
Cholesterol (mg/dl)	102.82	27.20	116.00	16.24	139.50	3.54	73.00	70.71

Notes: Control = VD₃ sufficient, VDD = VD₃ deficient, M = male, ND = no disease, S.D. = standard deviation, HDL = High-density lipoprotein, LDL = Low-density lipoprotein. Red font indicates higher levels compared to control group.

Body weights of animals on day 1 of experiments 1 and 2 were compared. The mean body weights (g) for male vitamin sufficient (21.58 ± 1.95) versus VDD (21.5 ± 1.99) mice, and female vitamin sufficient (17.83 ± 0.99), VDD (17.09 ± 1.35) mice were not different from control. In addition, bone vascularity, trabecular volume, and osteoblast and osteoclast numbers were evaluated on the same HE-stained longitudinal sections of tibia and no significant differences between the control and VDD group were observed.

Bone Marrow and Spleen BCR-ABL Leukemia Burden is Lower in VDD Mice Compared to Vitamin D Sufficient Mice

To further assess the relative leukemia burden at the primary sites of leukemia replication such as bone marrow and spleen. We quantified the number of luciferase-tagged BCR-ABL cells in the bone marrow (**Figure 4-9A**). In addition, a blind scoring of the disease burden in H&E stained histological sections of the hind limb and spleen was performed by the pathologist (Dr. Laura Janke, St. Jude Children's Research Hospital) (**Figure 4-9B, 4-9C**). Consistent with lower total leukemia body burden (**Figure 4-6**), VDD male mice had lower levels of bone marrow, hind limb and spleen leukemia.

Mice Vitamin D Deficient for a Shorter Time Interval Still Show Increased Survival from BCR-ABL ALL Compared to Vitamin D-Sufficient Mice

All of the mouse survival studies were performed on mice rendered vitamin deficient by placing pregnant females on vitamin D deficient diets during pregnancy and parturition and maintaining weaned pups on these deficient diets. We also used an alternate strategy to generate vitamin D deficiency in a shorter time frame and compared BCR-ABL ALL survival between vitamin D sufficient mice and mice rendered VDD by placing them on a VDD diet at 4 weeks (weaning), using them after 10 weeks of age. Despite the shorter duration of vitamin D deficiency, VDD mice still had prolonged survival from BCR-ABL Arf^{-/-} ALL (**Figure 4-10**).

1,25(OH)₂VD₃ Increases the Number of BCR-ABL Arf^{-/-} ALL Cells Only When Co-cultured with Bone Marrow Stromal Cells

To begin exploring the mechanism of how BCR-ABL⁺ ALL was proliferating more rapidly in vitamin D sufficient versus deficient mice (**Figures 4-4, 4-5, 4-6**), we examined the effect of 1, 25-(OH)₂VD₃ treatment on BCR-ABL Arf^{-/-} leukemia cells. Because mouse BCR-ABL⁺ Arf^{-/-} leukemia cells, like human primary B-ALL cells (Consolini et al., 2001), lack VDR (**Figure 4-11**) required for mediating the VD₃ signal, and because leukemic cells proliferate in the bone marrow, BCR-ABL⁺ B-ALL cells were co-cultured with immortalized human mesenchymal stromal cells (hMSCs) expressing VDR (**Figure 4-11**) that mimic a bone marrow micro-environment. There was no effect of 1, 25-(OH)₂VD₃ on number of viable BCR-ABL⁺ B-ALL cells grown without

Figure 4-9. Luminescence measure and leukemia score in control and VDD male mice.

Notes: (A) Luminescence measure of BCR-ABL Luc⁺ Bone marrow disease burden in male control and VDD mice, (B) Hind limb leukemia score in male control and VDD mice, (C) Spleen leukemia score in male control and VDD mice.

Figure 4-10. Kaplan meier survival curves using male mice started on control (vitamin D sufficient) and VDD diet beginning at weaning.

Notes: (A) Study 3 - Control (n=15) versus VDD (n=15), (B) Study 4- Control (n=15) versus VDD (n=15), (C) Study 3 and 4 combined curves. Differences between survival curves was determined using Gehan-Breslow-Wilcoxin test. The median survival for the control mice vs. VDD mice was 26 versus 36 days (A) and 26 versus 30 days (B).

Figure 4-11. Determination of VDR expression using PCR.

Notes: (A) Lane 1: M.W, Lane 2-6 showing GAPDH band at 500bp and lane 2-4 (positive control: mouse duodenum, kidney) mVDR band at 300bp, no band in lane 5-6 for mVDR in BCR-ABL ALL cells, (B) Lane 2-4 showing GAPDH band at 500bp and lane 5 (positive control, LS180 cells) hVDR band at 190bp, lane 6: hMSCs treated with ethanol no band of hVDR, lane 7: hMSCs treated with 1,25(OH)₂VD₃ a band of hVDR.

hMSCs ($p=0.5972$); however, 1, 25(OH) $_2$ VD $_3$ increased the number of BCR-ABL cells (2.102 ± 0.415 - fold higher, $p=0.012$) (**Figure 4-12**) when co-cultured with hMSCs. This result suggests that 1,25(OH) $_2$ VD $_3$ is acting through the stroma to enhance BCR-ABL ALL cell growth. In total, this result showed that 1, 25(OH) $_2$ VD $_3$ enhances BCR-ABL ALL cell number *in vitro* and this requires co-culture with bone marrow stroma.

1, 25(OH) $_2$ VD $_3$ Increases BCR-ABL Cell Migration to hMSCs in Transwell Culture

Since the *in vivo* vitamin D sufficient (control) mice showed higher ALL disease burden in the bone marrow, compared with the VDD mice (**Figure 4-9**) we next tested whether this also reflected vitamin D increasing migration of the leukemic blasts to the bone marrow stroma. An *in vitro* cell homing assay was used in which we measured the percentage of BCR-ABL leukemia cells migrating from the donor chamber of a transwell culture through a semi-permeable membrane to the bottom chamber containing media or containing hMSC conditioned with or without 1,25(OH) $_2$ VD $_3$ (**Figure 4-13**).

There was no effect of 1,25(OH) $_2$ VD $_3$ on migration of ALL cells in the absence of hMSCs. However, BCR-ABL ALL cells were stimulated to migrate to hMSCs and a significantly greater percentage of BCR-ABL cells migrated to hMSCs conditioned for 48 hours with 100 nM 1, 25-(OH) $_2$ VD $_3$ (**Figure 4-13**, $**p=0.007$). Fetal bovine serum (FBS), which alone can also provide the media with factors required for BCR-ABL cell migration, further increased the effect of 1, 25-(OH) $_2$ VD $_3$ ($p = 0.064$). This result demonstrates the ability of 1, 25-(OH) $_2$ VD $_3$ to enhance BCR-ABL cell migration is not intrinsic to the BCR-ABL blast, but rather, is due to VD $_3$ -initiated reprogramming of the hMSCs to attract more BCR-ABL cells.

1,25(OH) $_2$ VD $_3$ Increased CXCL12 mRNA and Protein Expression in hMSCs *in vitro* and in Mouse Serum *in vivo*

CXCL12 (stromal cell-derived factor-1, Sdf-1) is a chemokine produced by stromal cells, endothelial cells and osteoblasts, that plays an important role in normal B-cell lymphopoiesis, cell trafficking and homing to the bone marrow (L. Ding & Morrison, 2013), and it has been shown to increase proliferation of primary pre-B-ALL (Mowafi et al., 2008). Induction of CXCL12 mRNA (**Figure 4-14**) expression was higher in hMSCs treated with VD $_3$ for either 48 or 72 hours compared to control vehicle treated cells (9.179 ± 2.415 -fold ($****p < 0.0001$) and 10.320 ± 2.060 -fold ($***p = 0.0002$) increases, respectively.

Likewise, cell culture supernatants from hMSCs conditioned for 72 hours with 1,25-(OH) $_2$ VD $_3$, had a 2-fold increase in secreted CXCL12 protein compared with vehicle treated controls (**Figure 4-15A**, $*p=0.029$). There was a trend toward a higher serum CXCL12 protein level in VD $_3$ sufficient versus VD $_3$ deficient mice (**Figure 4-15B**, $p=0.095$). However, we did not find a significant difference in the CXCR4 (the

Figure 4-12. 1, 25(OH)₂VD₃ increases BCR-ABL proliferation in vitro.

Notes: The effect of 1,25(OH)₂VD₃ vs. vehicle treatment on growth of BCR-ABL ALL cell number when cultured in the absence or presence of hMSC and plotted as fold change relative to group control, n=3 per group. The unpaired t-test was used to compare differences in BCR-ABL cell number.

Figure 4-13. Increased migration of BCR-ABL leukemia cells to VD₃ treated bone marrow stroma.

Notes: The percentage of the total number of BCR-ABL ALL Luc⁺ cells that migrated to the bottom chamber was determined. The unpaired t-test was used to compare increase in cell number (**p<0.01).

Figure 4-14. Vitamin D induces CXCL12 mRNA in bone marrow stroma in vitro.

Notes: CXCL12 mRNA transcripts in hMSC cells treated with ethanol (EtOH) or 100 nM 1,25(OH)₂VD₃, n=8 or 10 per group. Unpaired t-test was used to determine significance between the groups (***p<0.001, ****p<0.0001).

Figure 4-15. Vitamin D induces CXCL12 protein expression in bone marrow stroma in vitro and in serum of mice in vivo.

Notes: CXCL12 protein expression in the media of hMSC cells treated with ethanol (EtOH) or 100 nM 1,25(OH)₂VD₃, n=4 per group. CXCL12 protein expression in the serum from VD₃ sufficient (control) or VDD mice, n=5 per group. Mann-Whitney nonparametric test on GraphPad was used to determine significance between the groups (*p<0.05)

CXCL12 receptor) expression in 1, 25-(OH)₂VD₃ treated versus ethanol treated BCR-ABL ALL cells (not shown). Hence, 1, 25-(OH)₂VD₃ reprograms the bone marrow stroma to produce and secrete CXCL12 to attract leukemia cells.

CXCL12 Increases the Number of BCR-ABL Cells in Suspension Culture

To determine whether CXCL12 could directly affect BCR-ABL cells, we treated cells in culture with CXCL12. Since VD₃ stimulated hMSC cells produced 0.5 ng/ml of CXCL12 (**Figure 4-16**), BCR-ABL cells in suspension culture, or co-cultured with hMSCs, were treated with 0.5 ng/ml CXCL12 for 48 hours. CXCL12 produced a significant increase (*p=0.04) in BCR-ABL cell number in cells in suspension culture, but no further increase in the number of viable BCR-ABL cells co-cultured with hMSC. This demonstrates that exogenous CXCL12 can directly stimulate growth of BCR-ABL leukemia cells.

Vitamin D Plus ALL Reprograms the Bone Marrow Stroma

A growing body of literature demonstrates a role for VD₃ in remodeling stroma that can then affect cancer growth and chemotherapeutic response (Mowafi et al., 2008). Similarly, the presence of leukemia in the bone marrow has been shown to transform the bone marrow niche into a leukemia-permissive microenvironment causes reprogramming (B. Kumar et al., 2018).

To test whether vitamin D remodels the extracellular matrix production by bone marrow stroma, immunohistochemistry for type I and V collagen was performed on the bone marrow of healthy vitamin D sufficient and deficient mice, as well as on those with BCR-ABL ALL obtained at the time of sacrifice. Both type I and V collagen were minimally detectable in the bone marrow of healthy mice, regardless of vitamin D levels, but increased in the marrow of mice with ALL, but more frequently in the VD₃ sufficient (control) group (**Figure 4-17**). Hence, the presence of BCR-ABL together with vitamin D is reprogramming the bone marrow stroma to increase production of type I and V collagens.

Vitamin D Deficiency Alters the Number of CXCL12 Expressing Cell Types in Bone Marrow and Bone

To evaluate if vitamin D deficiency had any effect on the bone marrow microenvironment, we evaluated the abundance of different cells that express CXCL12 in bone tissue and bone marrow. In vitro vitamin D induced CXCL12 expression in hMSCs, but in vivo many other cells types such as endothelial cells, osteoblasts, perivascular stromal cells and CXCL12 abundant reticular cells (CAR) also significantly express CXCL12. We analyzed the percentage of these cell types by flow cytometry in vitamin D deficient versus sufficient mouse bone and bone marrow tissues. We observed that, VD₃

Figure 4-16. CXCL12 increases proliferation of BCR-ABL ALL cells in suspension culture.

Notes: Effect of CXCL12 treatment (48 hr) on BCR-ABL cell number in cells cultured alone or with hMSCs, $n=3$ per group. The unpaired t-test was used to compare increase in cell number (* $p<0.05$)

Figure 4-17. Effect of vitamin D and BCR-ABL leukemia on expression of type 1 and type V collagens in mouse bone marrow.

Notes: Fluorescent immunohistochemistry was performed on paraffin embedded mouse tibias to examine the expression of collagens 1 and V *in vivo*. Collagens 1 and V both show the highest expression in VD₃ sufficient mice with ALL.

deficient mice had lower percentage of CD31 negative cells (**Figure 4-18**) in the bone marrow which consists of all the CXCL12 expressing cells except endothelial cells. The percentage of CXCL12 expressing cell types P α S stromal cells (P α S), mesenchymal stem cells (MSCs), osteoblasts and CAR cells in bone marrow was low in the bone marrow of vitamin D deficient mice compared to the sufficient mice. We also observed a significant effect of vitamin D deficiency in the bone tissue where CD31 negative cell percentage was significantly low compared to sufficient mice (**Figure 4-19**). However, P α S, MSCs and osteoblasts, although they make up a very small percentage of CD31 negative cells, were significantly higher in VD₃ deficient mice compared to sufficient mice. But CARs are the most abundant CD31 negative cells making up to 25-30% of CD31 negative cells and are significantly lower in VD₃ deficient bones (**Figure 4-19**).

Discussion

Despite the high frequency of vitamin D deficiency in humans worldwide, this is the first study to test and demonstrate that host deficiencies in vitamin D can affect growth of BCR-ABL ALL Arf^{-/-} leukemia and survival of mice from this disease. Our study found that vitamin D sufficient mice had markedly accelerated disease progression and decreased survival from BCR-ABL ALL compared to mice that were vitamin D deficient. A similar effect of vitamin D deficiency on survival from BCR-ABL ALL was seen regardless of whether mice were exposed to a VDD diet during gestation (**Figure 4-7**) or started the diet at weaning (**Figure 4-10**), demonstrating the effect vitamin D deficiency on BCR-ABL leukemogenesis in this mouse model does not require deficiency during gestation.

Surprisingly, host VD₃ sufficiency versus deficiency was detrimental to host survival from BCR-ABL Arf^{-/-} ALL. Importantly, 1,25 (OH)₂VD₃ had no effect on growth of the leukemia cells in suspension culture but required co-culture with the bone marrow stroma. This result is consistent with the apparent absence of the VDR in mouse BCR-ABL⁺ ALL Arf^{-/-} leukemia cells, and similarly its absence in human primary B-ALL cells (Consolini et al., 2001).

Our results also offer a cautionary reminder for the many studies that test the effect of chemicals on leukemia cells grown in suspension culture, without co-culture of bone marrow stromal cells, as these studies may miss important leukemia cell responses that are only seen in a co-culture setting. Bone marrow stroma cells are known to form a microenvironment not only conducive for leukemic cell growth, but that can also regulate responsiveness to anti-leukemic therapies such as asparaginase and tyrosine kinase inhibitors (Iwamoto et al., 2007; B. Zhang et al., 2013).

Our results extend this paradigm to the effect of 1, 25-(OH)₂VD₃ on growth and migration of BCR-ABL⁺ ALL Arf^{-/-} leukemia cells. 1, 25-(OH)₂VD₃ actively induced bone marrow stroma to release CXCL12 in vitro, and increased CXCL12 serum levels in vivo, resulting in an increased ability to attract the BCR-ABL⁺ Arf^{-/-} ALL to the marrow and support its growth. Different cell types in the bone marrow, other than hMSCs that

Figure 4-18. Percentage of different CXCL12 expressing cells in bone marrow of VD₃-sufficient mice versus VD₃-deficient mice.

Figure 4-19. Percentage of different CXCL12 expressing cells in bone tissue of VD₃-sufficient mice versus VD₃-deficient mice.

produce CXCL12 may also be responsive to 1, 25-(OH)₂VD₃ and increase the production of chemokine CXCL12. Apart from hMSCs, *in vivo* multiple other cell types such as PaS mesenchymal stem cells, osteoblasts, endothelial cells and CARs express CXCL12.

Above all, CARs express significant amount of CXCL12 in the bone marrow microenvironment. In bone and bone marrow of vitamin D sufficient mice CAR cell percentage is significantly higher compared to VD₃ deficient mice. Our results suggest that vitamin D not only can induce CXCL12 expression *in vitro*, but *in vivo* vitamin D can significantly affect the number of CXCL12 expressing cells.

In vivo, vitamin D, in the presence of BCR-ABL+ Arf-/- leukemia, also remodeled the bone marrow stroma, inducing expression of Type 1 and V collagens. Others have reported that increased bone marrow reticulin and collagen fibers are associated with malignant diseases and may anchor and trap the leukemic cells in the marrow providing an environment where they become more drug resistant (Nath et al., 2011). While this study probed the interaction of 1, 25-(OH)₂VD₃ signaling through the co-cultured bone marrow stromal cells, *in vivo* multiple other cell types in the bone marrow express VDR, including osteoblasts. Hence, *in vivo*, Vitamin D may signal through VDR in additional cells in the bone and bone marrow influencing the microenvironment and differentially altering the growth and viability of the leukemia cells.

It will be important to determine if vitamin D levels significantly affect the growth of BCR-ABL+ Arf-/- ALL in mice *in vivo* can be extended to other subtypes of pediatric leukemia. Moreover, do these results in mice translate to humans? Interestingly, a study in children that measured dietary vitamin D intake in newly diagnosed children with ALL found that vitamin D intake was significantly different between patients who presented with high-risk ALL versus standard risk ALL. Failure to meet daily dietary amounts of vitamin D was higher in the standard risk versus high-risk patients, while the proportion of patients who met the vitamin D dietary requirements was higher in the high risk versus standard risk patients (Ladas et al., 2016). Clearly our findings suggest that the clinical levels of vitamin D should be measured in leukemic patients over the course of therapy to determine if there is an association in humans between vitamin sufficiency and survival from different subtypes of leukemia, and if vitamin supplementation to vitamin deficient patients affects survival from acute lymphoblastic leukemias.

CHAPTER 5. SUMMARY

The overall goal of this research work was to evaluate if vitamin D deficiency could cause any vitamin – drug interaction with CYP3A4 substrates and if it has an effect on survival outcome from B-ALL. We developed diet-based age-controlled vitamin D sufficient and deficient mice and evaluated changes associated with vitamin D status on intestinal, hepatic and renal drug metabolizing enzymes (CYP3A) and drug transporter (PGP, BCRP, MRP4). We found that dietary vitamin D can alter mouse intestinal Cyp3a11 and hCYP3A4 expression in female mice. Paradoxically, we also observed a significant increase in intestinal transporter expression in both male and female vitamin D deficient mice. However, these changes did not cause a significant vitamin drug interaction with dexamethasone and dasatinib which are both CYP3A4 and PGP substrates and given orally to ALL patients. However, we found that vitamin D sufficient mice had markedly accelerated disease progression and decreased survival from BCR-ABL ALL compared to mice that were vitamin D deficient.

DEX is a potential inducer of CYP3A depending on the concentrations achieved in the tissue. We calculated gut concentrations of DEX to be in sub-micromolar range and can activate PXR to induce CYP3A genes. Utilizing intestinal and liver tissues from DEX treated mice for 3.5 days, we analyzed the expression of CYP genes. Our results suggest that, 3.5 days treatment of DEX has significantly induced multiple CYP3A genes in the intestine of vitamin D deficient group but not the sufficient group. Moreover, we analyzed the expression of CYP3A and CYP2B genes in the livers of DEX treated mice. The plasma concentrations of DEX in this study was in nanomolar range, which can activate GR but not PXR. We observed significant induction of CYP2B genes in all the groups indicating GR activation in the livers.

When we placed vitamin D sufficient and deficient mice injected with BCR-ABL ALL cells on discontinuous DEX regimen, despite the treatment, vitamin D sufficient mice fared worse than the vitamin D deficient mice. This indicated that vitamin D itself has an effect on BCR-ABL leukemia progression. Therefore, we next evaluated the mechanisms by which vitamin D can affect leukemia cells.

Our studies show that BCR-ABL ALL cells do not express VDR, a receptor through which vitamin D binds and regulates gene transcription. However, many cells in the bone marrow microenvironment in which the lymphoblasts proliferate *in vivo*, such as osteoblasts, endothelial cells and CARs express VDR. *In vitro* the BCR-ABL ALL cells are co-cultured in with hMSCs, which act as a bone marrow micro-environment, and these cells do express VDR. Co-culturing BCR-ABL cells with hMSCs and treating with vitamin D directly increased the proliferation of BCR-ABL ALL cells and increased their migration to co-cultured hMSCs. The increased migration of BCR-ABL ALL cells to hMSCs was mediated by Vitamin D induction of CXCL12, an important chemokine for leukemia cell proliferation and migration to hMSCs. *In vivo*, vitamin D, in the presence of BCR-ABL+ Arf-/- leukemia, also remodeled the bone marrow stroma, inducing expression of Type 1 and V collagens, and further increased the number of CARs

(CXCL12 producing cells) in the bone and bone marrow. Consequently, Vitamin D may signal through VDR in neighboring supportive cells in the bone and bone marrow, remodeling the microenvironment and differentially altering the growth and viability of the leukemia cells.

Our studies have highlighted that vitamin D deficiency has a significant effect on survival outcome. Although many retrospective reports suggest the association of vitamin D levels and cancer risk, since their primary end point was not overall survival or mortality, it will be important to design individual clinical trials to ascertain the association of vitamin D levels and survival outcome. Since many pediatric leukemia patients are given vitamin D supplementation, it is important to understand the impact of VD₃ supplementation during chemotherapy on survival and drug efficacy in patients with pre-B cell ALL. Further efforts are needed to elucidate vitamin D's role and potential utility in the clinical setting for different leukemia subtypes.

Clearly our findings suggest that the clinical levels of vitamin D should be measured in leukemic patients over the course of therapy to determine if there is an association in humans between vitamin sufficiency and survival from different subtypes of acute lymphoblastic leukemia, and if vitamin supplementation to vitamin deficient patients affects survival from acute lymphoblastic leukemias.

With vitamin D having a significant effect on BCR-ABL ALL cell proliferation and migration, it will be important to determine if vitamin D levels also affect other subtypes of pediatric leukemia. Moreover, there are many studies suggesting different roles of vitamin D in mice versus humans. Thus, it will be important to study if these results in mice translate to humans.

Our data demonstrate that dietary vitamin D can influence intestinal mouse Cyp3a11 and human CYP3A4 expression. Given that the level of induction of CYP3A genes is different between vitamin D deficient and sufficient mice, further studies investigating possible drug-drug interactions that could arise when CYP3A4 drugs are co-administered with DEX in vitamin D deficient condition could be of significant importance. This data might help in better understanding of clinical situations where multiple drugs are co-administered during various stages of chemotherapy.

The broad expression pattern and widespread effects of vitamin D signaling can lead to unexpected outcomes and caution might be needed while supplementing patients with vitamin D. Owing to the known pleiotropic roles of vitamin D, and taking into consideration unexpected results from this study showing vitamin D deficient mice have a survival advantage from ALL, as mentioned before it is important to study further effects of vitamin D deficiency and supplementation in other B-cell leukemia subtypes and other diseases. Vitamin D was shown to cause stromal cell remodeling in pancreatic cancer (Sherman et al., 2014), fibrosis in hepatic stellate cancer (N. Ding et al., 2013) and in this study altering the bone marrow microenvironment suggesting an indirect role of vitamin D on disease outcome. Although it has been reported that high vitamin D levels or intake of vitamin D reduces the risk of colorectal, breast cancer as well as type I

diabetes, multiple sclerosis and hypertension (Holick & Chen, 2008), it is critical to reevaluate any effects of vitamin D status on the disease progression and outcome as well as to discern the mechanistic pathways altered by vitamin D status which directly or indirectly can affect the disease outcome. This would be best performed through clinical trials that would evaluate, first, whether vitamin D status (without supplementation) affects disease progression or survival across ALL subtypes before beginning any vitamin D therapeutic treatment in these patients.

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